Uncoupling Lipid Metabolism from Inflammation in Adipose Tissue

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Hongliang Xu

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Advisor: David A. Bernlohr, Ph.D

August 2016

Acknowledgements

I would like to thank my advisor, Dr. David Bernlohr for giving me a “home” for four splendid years. I will forever be grateful for the support and guidance Dr. Bernlohr has given me over the years. Not only those, his wisdom and rational thoughts on carrying out scientific projects will continue to guide me through my research career.

I would also like to thank Dr. Ann V. Hertzel for her great support and thoughtful comments on my projects over the years. She has not only been a great friend, but also a wonderful trainer at the early stage of my career. Work and collaborate with her has been one of the most wonderful things in the last few years.

Additionally, I would like to give my special thanks to my collaborator and friend, Dr. Cyrus Jahansouz. Cyrus joined the lab slightly more than two years ago but his passion and wisdom on research made us intimate friends and collaborators in no time. Work with Dr. Jahansouz has been a dream journey, and I believe, will continue to be fruitful in future.

Finally, I would like to thank Dr. Rocio Foncea Avila, Wendy Hahn, Dr. Anna Kristina Hellberg, Dr. Joe Burrill, Dr. Jovan Kuzmicic, Kaylee Steen, Amy Hauck, Ajeetha Rajan and many other present and past members of Bernlohr’s lab for the great support over the years.

Dedication

This thesis is dedicated to my PI Dr. Bernlohr and everyone from Bernlohr's lab that helped my throughout the journey of graduate school.

To my master program mentor from Capital Normal University, Dr. Yingkao Hu, who has been always supportive and encouraging on my research in the new field diverted from plant biology.

To my friends and former colleagues Dr. Ke Wang, Dr. Yinan Kan, Dr. Li Ou, Yu Zhang for always being someone I can count on.

To my family, thanks for the great understanding and support on my career and life.

Abstract

Obesity has become an epidemic that affects the quality of life for more than one third of the US population. Syndromes associated with obesity, such as type 2 diabetes, fatty liver disease, cardiovascular disease, and hyperlipidemia cost billions of dollars to health care annually. One such major obesity-linked syndrome is the development of insulin resistance, which can lead to other systemic complications. Studies conducted over the years indicate insulin resistance is exacerbated due to increased infiltration and chronic activation of immune cells into adipose tissue as well as many other tissues such as liver, skeletal muscle and pancreas. Therefore, targeting immune cells to reduce inflammation is an important weapon to treat obesity related syndrome. Interestingly, deficiency of fatty acid binding protein 4 (FABP4, also know as aP2) in macrophages suppresses inflammatory activation of the cells and contributes to whole body insulin sensitivity in an animal model. However, the mechanism of how FABP4 deficiency leads to the suppressed inflammatory was not well defined. In my thesis research, we identified uncoupling protein 2 (UCP2) and Sirtuin 3 (Sirt3) as important mediators of the anti-inflammatory phenotype in FABP4 deficient macrophages. Increased expression of UCP2 in FABP4 null macrophages contributes to both suppressed inflammation and endoplasmic reticulum stress (ER stress) by reducing mitochondrial production of reactive oxygen species (ROS), while Sirt3 induction contributes to suppressed inflammation, increased beta-oxidation, and improved mitochondrial function in

FABP4 deficient macrophages. Interestingly, the FABP4-UCP2 axis also extends to a human bariatric surgery model. In the subcutaneous adipose tissue from patients undergoing bariatric surgery, decreased expression of FABP4 and concomitant increase of UCP2 expression was observed seven days post surgery. The increased expression of UCP2 reduced oxidative stress in the tissue and may contribute to the early on metabolic improvements in these patients.

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CHAPTER ONE

Chronic Inflammation and Oxidative Stress in The Development of Insulin Resistance

Hongliang Xu wrote this chapter in its entirety.

From Obesity to insulin resistance- the role of chronic inflammation

Obesity has become an epidemic that affects close to one third of the American population (1). From an evolutionary point of view, storage of excess nutrients in the form of triglycerides in adipose tissue is a fitness function that helps mammals survive adverse periods of drought and famine. From this perspective, as survivors of the evolutionary selection, modern-day humans are prone to lipid storage and the development of obesity (2). Despite the benefits of expanding adipose tissue, prolonged storage of excess lipids can negatively affect the normal function of many organs, such as liver, muscle, pancreas, heart, and the central nervous system (3-5). This broad impact of aberrant lipid storage leads to the increased incidence of many pathological conditions associated with obesity including type 2 diabetes mellitus (T2D), hypertension, fatty liver disease, cardiovascular diseases (CVD), and many types of cancer (3, 4).

Development of obesity, from a widely accepted perspective, is accumulation of excess fat tissue, mostly white adipose tissue (WAT). Therefore, understanding adipose tissue biology is essential and the first step to resolve many obesity related diseases. White adipose tissue is roughly divided into two physiologically different categories: subcutaneous adipose tissue and visceral adipose tissue (3). In contrast to the original dogma describing adipose tissue as a passive depot for lipid storage, visceral WAT, in particular, has been proven to be an active endocrine organ that secretes a huge panel of cytokines, including leptin,

adiponectin, tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1), fatty acid binding protein 4 (FABP4), and many others (3, 5-7). These cytokines are often referred to as adipokines, and play important roles in regulating tissue homeostasis of not only adipose tissue but many other tissues (8, 9). For instance, leptin can exert its effect on the central nervous system and regulate appetite. The leptin deficient mouse model (referred to as ob/ob mouse) is one of the most commonly used models to study obesity related syndromes (10, 11). Ob/Ob mice become extremely obese even on regulator diet due to uncontrolled food intake, leading to the development of hyperinsulinemia, hyperglycermia, and fatty liver diseases. This collectively indicates the importance of this adipokine on regulating whole body metabolic homeostasis (10-12). Adiponectin is another adipokine primarily made by adipose tissue and is involved in regulation of glucose metabolism and fatty acid oxidation in multiple tissues, such as the central nervous system (CNS), liver and skeletal muscle (13, 14). Deficiency of adiponectin signaling in mice predisposes the animals to a variety of characteristic obesity-linked metabolic syndromes, such as insulin resistance, hyperlipidemia, and cardiovascular diseases (15-18). Given the fact that development of obesity leads to decreased expression of adiponectin, the adipokine has become a pharmaceutical target for treating obesity related metabolic syndrome (19-21).

With the knowledge that adipose tissue plays an important role in energy storage and functions as an active endocrine organ, it is important to ask how does expansion of adipose tissue related to metabolic syndrome? A critical process connecting obesity to metabolic disease is the development of insulin resistance (3, 5, 22-24). As shown in figure1, obesity is accompanied with size expansion of adipocytes and increased basal lipolysis, which leads to increased secretion of free fatty acids (FFA) and certain adipokines secretion into circulation. FFA and the adipokine, MCP1, can serve as chemotaxins to recruit immune cells such as T cells, dendritic cells, eosinophils and macrophages, which infiltrate into adipose tissue (25, 26). These immune cells can be activated in adipose tissue and secrete a panel of inflammatory cytokines such as IL6, TNFa, interleukin 1β (IL1β) and MCP1, and together with the adipokines, promote insulin resistance in adipose tissue (3, 23, 26, 27). Additionally, these cytokines from adipose tissue can circulate in serum and cause insulin resistance in many other tissues such as brain, liver, and skeletal muscle (28, 29). Global insulin resistance will not only leads to ectopic lipid deposition in nonadipose organs, such as liver and skeletal muscle, but also cause hyperinsulinemia. High level of insulin will eventually lead to pancreatic β cell failure and onset of T2D (29).

As mentioned above, immune cell infiltration is an important step for the development of insulin resistance in adipose tissue (25). In fact, increased immune cell infiltration also happens in other organs where insulin resistance

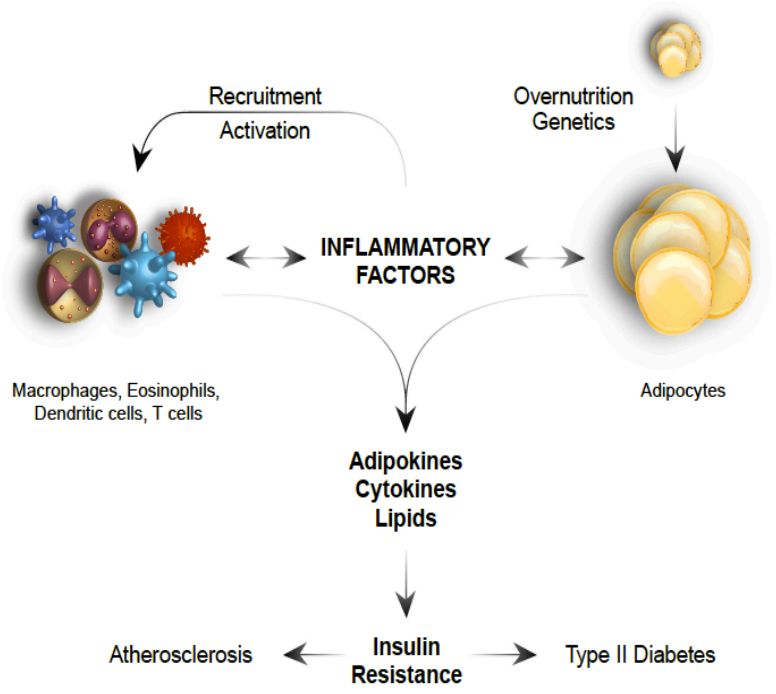

Figure 1 - Adipocyte centric development of obesity induced insulin resistance.

Credit to Tony Hertzel for creation of this figure for using by the Bernlohr Lab.

develops, such as CNS, liver and muscle (30). After tissue infiltration, these immune cells, such as dendritic cells, T cells, and macrophages are polarized to a chronically, low grade activation state and causing chronic inflammation in different tissues (22, 30). Nowadays, this low-grade chronic inflammation has become a hallmark and recognized as a major contributor to obesity-induced insulin resistance (3, 22, 30).

Interestingly, the first observation that linked inflammation to insulin resistance was made in adipose tissue by Hotamislgil et al. about twenty years ago, when they demonstrated that neutralization of TNFα in a diet induced obese mouse model, regained insulin sensitivity (31). Multiple studies have gone on to further show the increased accumulation of immune cells, especially macrophages in both human and rodent adipose tissue (32, 33). In fact, macrophages can account for up to 40% of total cells in visceral adipose tissue of obese subjects (32, 33). One distinct feature of macrophages in obese adipose tissue is that they are primarily polarized to a proinflammatory state, as evidenced by a heightened array of proinflammatory gene expression, including TNFα, IL6, MCP1, resistin, IL1β, and plasminogen activation inhibitor 1 (PAI1) (34, 35). These macrophages are also referred to as classically activated macrophages (M1 macrophages). In contrast, the non-inflammatory polarized macrophages, which are referred to as

alternatively activated macrophages (M2 macrophages), mainly secrete anti-inflammatory cytokines such as interleukin 10 (IL-10), and IL4. M2 macrophages are also less abundant in adipose tissue of obese subjects (36). Multiple labs have repeatedly demonstrated the importance of the predominance of M1 over M2 macrophages in causing insulin resistance (37-39).

Although macrophages are the most abundant proinflammatory immune cells infiltrating adipose tissue and liver, other types of immune cells do play an important role in inflammation induced insulin resistance. For instance, $CD8^+$ cytotoxic T cells, and $CD4^+$ Th1 cells have been shown to promote insulin resistance (40-42). On the contrary, $CD4^+$ regulatory T cells (T-regs) can exert an anti-inflammatory effect by inhibiting proinflammatory polarization of macrophages and further recruitment of circulating macrophages into the tissues (43). The number of T-regs is decreased in adipose tissue of high fat fed mice but can be pharmacologically restored by treating the mice with Thiazolidinedione (TZD) and ultimately sensitizing the mice to insulin signaling (40, 44, 45). Another important immune cell population in maintaining tissue insulin sensitivity is eosinophils. Adipose tissue ablation of eosinophils has been shown to induce insulin resistance (46). Furthermore, neutrophils and mast cell numbers increase with obesity and also contribute to diet induced insulin resistance (47, 48). Given the diverse roles of immune cells in inflammation and

regulation of insulin sensitivity, the next important question is how does inflammation lead to insulin resistance?

Inflammation and insulin resistance converge on the activation of two transcription factor signaling pathways. The first of these two is the NF-κB pathway, which is activated by inhibitor of NF-κB kinase (IKK) (49-52). Interestingly, IKK can directly phosphorylate insulin receptor substrate 1 (IRS1) at a serine residue, inhibiting its activity (53). Also, activation of the NF-κB pathway leads to increased expression of downstream targets, TNFα, MCP1, IL6, IL1β, etc (52). Once secreted, TNFα binds to its receptor, which is expressed on both macrophages and adipocytes, leading to direct activation of IKK and a feed-forward mechanism of inflammation. MCP1 expression from M1 macrophages, which is far more abundant compare to that from the adipocyte, can recruit more macrophages into the tissue (54). Not surprisingly, inhibition of NF-κB pathway via genetic means or pharmacologically in animal models have been shown to improve obesity induced insulin resistance (49, 55, 56). The other important pathway that connects inflammation to insulin resistance is the c-Jun NH_2-terminal kinase (JNK) pathway (57, 58). Similarly to IKK, JNK can also inhibit insulin signaling by serine phosphorylation of IRS1. This observation was supported through genetic crossing of JNK1 knockout mice to ob/ob mice, which protected the animal from insulin resistance (59, 60).

Suppress inflammation to improve insulin sensitivity- PPARγ as a potential drug target

Development of obesity leads to increase of chronic inflammation, which results in insulin resistance. Therefore, targeting inflammation has been an important solution to improve insulin sensitivity for treating obese and diabetic patients.

One important drug target for treating diabetic patients is Peroxisome proliferator activated receptor γ (PPARγ). PPARγ belongs to a subset of nuclear receptor superfamily, which also include the other two members PPARα and PPARβ/δ (61-63). PPARγ is highly expressed in adipose tissue, and also expressed in skeletal muscle, liver, osteoclasts, and many immune cells (64). Studies with various genetic models have indicated PPARγ plays an important role in glucose and lipid metabolism (65-67). Not surprisingly, whole body knockout of PPARγ is lethal (64). As a nuclear hormone receptor, PPARγ is activated upon ligand binding (68). Once activated, PPARγ heterodimerizes with RXR (retinoic X receptor), translocates into the nucleus, and induces expression of downstream targets (68, 69). In adipocytes, PPARγ plays an important role in adipogenesis by up regulating the expression of Cluster of Differentiation 36 (CD36), FABP4, adiponectin, and CCAAT/enhancer binding protein α (70). Loss of function in PPARγ results in lipodystrophy in both humans and mice while overexpression of PPARγ in fibroblasts can induce an adipocyte-like phenotype (70). Tissue

specific deficiency of PPARγ in adipose tissue, muscle, and liver predispose mice to insulin resistance (69, 71-74). In human, a polymorphism of the *PPARγ* gene Rro12Ala is associated with improved glucose homeostasis while the dominant-negative mutations lead to severe insulin resistance (75, 76).

Thiazolidinediones (TZDs) are a class of drugs widely used as insulin sensitizer to treat type 2 diabetes patients (69, 77). In 1995, it was discovered that TZDs were high affinity ligands for PPARγ, and exert their insulin sensitizing effects, at least partially, through activation of PPARγ (78). Since adipose tissue has the highest expression of PPARγ, insulin-sensitizing effects of TZDs primarily work through the fat tissue. Indeed, mice lacking adipose tissue PPARγ are less responsive to the insulin-sensitizing effects of TZDs (71, 79). However, it is worth noting that adipose glucose disposal only contribute modestly to global glucose homeostasis effects of insulin, and muscle and liver are the major organs involved in insulin induced glucose disposal (80). Therefore, the effects of TZD action must also come from the communication between adipose tissue and other tissues. Actually, one such messenger from adipose tissue to other tissues is adiponectin. TZDs treatment increases expression of adiponectin while reducing adipocyte expression of a number of insulin resistance-promoting polypeptides (80).

Despite the insulin-sensitizing efficacy of TZDs as therapeutic drugs for treating T2D, there are indeed some complications observed in clinical application. As a target for TZDs, PPARγ has an adipogenic effect, yet increased adiposity is a risk factor for T2D (81). Also, PPARγ can be induced in fatty liver, which potentially explains the exacerbated hepatic steatosis observed in TZD treated diabetic mouse models (66). Indeed, over coming these side effects of TZD treatment has become a major challenge to the development of new and more specific drugs.

As briefly mentioned before, PPARγ is also expressed in immune cells such as dendritic cells, monocytes and macrophages (82). TZDs, including pioglitazone, troglitazone and rosiglitazone were all shown to suppress inflammation in immune cells (83-85). The inflammation suppressive effects of PPARγ activation also come from the report that mice with macrophage specific knockout of PPARγ were less susceptible to *Leishmania spp.* infection, indicating enhanced M1 macrophage function (86). Detailed studies revealed that loss of PPARγ in macrophages inhibit their differentiation into the non-inflammatory M2 state and has a broad effect on whole body metabolic status of the mice (86). Treatment of macrophages with TZDs has been shown to reduce the expression of inflammatory proteins expression such as TNFα, inducible nitric oxide synthase (iNOS), and Matrix metallopeptidase 9 (MMP9) (87). Mechanistically, it was indicated that ligand activated PPARγ can inhibit the inflammatory activity of NF-

κB, activator protein 1 (AP1), and signal transducers and activators of transcription 1 (STAT1) through direct interaction (85, 88).

The anti-inflammatory effect of PPARγ activation was also extended to other inflammatory diseases, such as atherosclerosis and inflammatory bowel disease (82, 89). When macrophages take up oxidized LDL, there can be massive cholesterol accumulation intracellularly, which is a hallmark for atherosclerotic lesions. Interestingly, TZD treatment dramatically reduced atherosclerosis in mouse models by both suppressing cytokine release and regulating cholesterol efflux from macrophages (82, 90). Importantly, human studies have also suggested the anti-atherogenic effects of TZDs (91). In addition to the anti-atherogenic effects of PPARγ, TZDs were also used in other clinical trails to treat inflammatory bowel disease for its anti-inflammatory effects (92, 93). Taken together, PPARγ serves as an important target for improving insulin sensitivity, at least partially by suppressing immune cell inflammation.

Suppress inflammation to improve insulin sensitivity- AMPK as a potential drug target

AMPK (AMP- activated protein kinase) is another important drug target for treating obesity and type 2 diabetes. Structurally, AMPK is a heterotrimeric complex with a catalytic α subunit and regulatory β and γ subunits. Each of the subunit has different isoforms expressed in different tissues and could form different complex combinations, which may influence the activity and subcellular localization of the enzyme complex (94, 95). The trimeric structure allows the enzyme complex to be regulated in different ways. The α subunit contains a conserved threonine residue (Thr172), which is the phosphorylation site for upstream kinases LKB1 (liver kinase B1), CAMKKβ (calmodulin-dependent protein kinase kinase β), or TAK1 (transforming growth factor β activated kinase 1) (96-98). Thr172 phosphorylation can increase the enzyme activity over 100 fold. The regulatory γ subunit contains four CBS (cystathioine-β-synthase) domains, one of which binds AMPK constitutively, and the other two can either bind AMP, ADP or ATP (99). However, AMP has a much higher binding affinity compared to ATP, allowing AMP to displace ATP in the γ subunit. Binding of AMP or ADP rather than ATP causes conformational changes that modulate the phosphorylation status of Thr172 (100, 101). The ratio of AMP/ATP can reflect the energy status of the cell, therefore, AMPK can sense the energy demand of the cell and regulate its targets accordingly (102).

AMPK activation can lead to phosphorylation of a number of key downstream targets to regulate carbohydrate and lipid metabolism, resulting in activation of energy production pathways such as fatty acid oxidation, glucose transport, mitochondrial biogenesis, while inhibiting energy consumption processes such as fatty acid synthesis, cholesterol synthesis, hepatic gluconeogenesis, and mTOR (mammalian target of rapamycin)-regulated protein synthesis (94, 103, 104). The role of AMPK in energy production leads to the hypothesis that activation of AMPK in metabolic tissues can be a valuable therapeutic strategy to treat obesity and insulin resistance (104). Indeed, metformin which believed to work through AMPK activation, is one of the most prescribed drugs to treat diabetes (105). More recently, salicylate was also shown to activate AMPK and have an anti-diabetic effect. Interestingly, the combined use of metformin and salicylate, which activate AMPK through different mechanisms, resulted in greater AMPK activation and greater efficacy in relieving insulin resistance than either drug alone (106).

A number of studies have also suggested an anti-inflammatory effect of AMPK activation in different tissues, which is a major reason for the anti-diabetic effect of drugs targeting the kinase. In macrophages, activation of AMPK can suppress the expression of inflammatory cytokines such as TNFα, IL6 and IL1β (107-110). Additionally, AMPK activation has been shown to induce M2 like macrophage cytokine IL10 production(108, 110). Furthermore, it has been shown that in type

2 diabetes patients, NLRP3 inflammasome activation is markedly increased. In contrast, metformin treatment reduced IL1β and IL18 secretion from monocyte derived macrophages, indicating an inflammasome suppression effect of AMPK (111). In mouse models, LPS (lipopolysaccharide) treatment of bone marrow derived dendritic cells dramatically reduced Thr172 phosphorylation of AMPK, while knockdown of AMPK increased the propensity of LPS induced activation in these cells (112).

Inflammatory activation of immune cells, such as dendritic cells, and macrophages, cause metabolic switches from oxidative phosphorylation to glycolysis, mimicking the Warburg effect in cancer cells (113). M1 macrophages, which were polarized by stimulation with LPS and interferon γ (IFNγ) have high accumulation of fructose-2,6-bisphosphate, thus increase glycolytic flux. On the other hand, IL4 and IL13 induced M2 macrophage polarization use oxidative phosphorylation as the major energy source and produce more anti-inflammatory cytokines (114). AMPK activation in macrophages drives this M2 polarization and switch metabolic to fatty acid metabolism (108). The evidence for this was shown in macrophages isolated from AMPK deficient mice where mitochondrial content was reduced, as well as the FA oxidation ratio, which is typical for M1 like macrophages (108).

In addition to the immune-suppressive effects on immune cells, AMPK also plays an important role in regulating inflammatory signaling in other cells types. For instance, knockdown of AMPKα in adipocyte cell line 3T3L1 increased the mRNA levels of TNFα and IL1β as well as fatty acids induced MCP1 expression (112). Furthermore, in high fat diet fed AMPKβ-/- mice, adiponectin levels were significantly decreased while leptin was increased (108). Taken together, AMPK serves as a sensor of nutrient status and plays an important role in regulating metabolic pathways and suppresses inflammation activation of both immune cells and other cell types, contributing to improvement of whole body insulin sensitivity. Therefore, targeting AMPK has been the focus for various anti-diabetic drugs (106).

From Obesity to insulin resistance- the role of Oxidative stress

Oxygen is essential for biological activities of eukaryotes in order to survive. However, biological processes involved in utilization of oxygen have the tendency to create reactive oxygen species (ROS) such as hydroxyl radical (•OH), superoxide radical ($O_2•^-$), hydrogen peroxide (H_2O_2), etc. (115). Under normal physiological conditions, a balance exists between production of ROS and the antioxidant capacity of the reducing equivalents such as glutathione, and peroxiredoxins (Prdx), or ROS scavenger enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidases (GPXs), and glutathione S-transferase (GST) (115). However, in obesity associated metabolic disorders such as insulin resistance, hyperinsulinemia, hypertension, and type 2 diabetes, the redox balance between ROS production and antioxidant capacity is compromised and increased oxidative stress is subsequently observed (116-119). Often times, oxidative stress can be both a trigger as well as an outcome of obesity. On one hand, oxidative stress can facilitate pre-adipocyte differentiation into mature adipocytes and increase adipose mass (120, 121). For instance, 3T3L1 cells treated with hydrogen peroxide induces differentiation even in the absence of insulin (122). In addition, genetic knock out of Prdx3, which is a major ROS scavenger, increase mice adiposity (123). Therefore, increased ROS promote the development of obesity related syndrome. On the other hand, increased adiposity is accompanied with increased adipocyte inflammation, fatty

acid oxidation, and accumulation of damaged mitochondria, all of which lead to further increase of ROS (121, 122, 124).

In order to target ROS to improve insulin sensitivity, the first question to answer is where the ROS are being generated. Based on the origin, ROS can be divided into mitochondrial ROS and non-mitochondrial ROS. In mitochondrial, ROS are mostly generated from different parts of electron transport chain (ETC) (115). Electrons enter into the ETC from TCA cycle intermediates via NADH (at complex I) and $FADH_2$ (at complex II). The transport of electrons is accompanied with proton movement from the mitochondrial matrix into intermembrane space and build up of a H^+ gradient across the inner membrane of mitochondria develops. Oxygen functions as the final electron receptor and produces water at complex IV of the ETC. The electron transport is coupled with production of ATP by complex V, which is driven by the proton gradient (125). However, this process is not perfectly coupled all the time, and if an electron prematurely leaks through complex I or III and reacts with oxygen, superoxide anion is generated. Superoxide is not stable and can be further converted to hydrogen peroxide by SODs (super oxide dismutase) (115). Therefore, ROS generation is a by-product of normal mitochondrial respiration. In addition to complex I and III, there are around 10 additional mitochondrial ROS generating systems identified to date (126). Importantly, two of these other systems may actually contribute to a significant portion of mitochondrial ROS production. This includes α-ketoglutarate

dehydrogenase and pyruvate dehydrogenase reactions (126). In addition to mitochondrial ROS, other organelles and enzyme complexes can generate ROS. For instance, the peroxisome consumes an appreciable amount of oxygen and is known to produce hydrogen peroxide (127). Moreover, a number of studies have shown that NADPH oxidase (NOX) activity correlate with increased oxidative stress in obesity (128-130).

In addition to inflammation, oxidative stress is another major cause for development of insulin resistance. This is partially because oxidative stress can activate stress responsive signaling such as MAP kinase (MAPK) cascades (131-133). Activation of MAPK leads to Ser/Thr phosphorylation of IRS, which not only suppressed its function but also promote its degradation (134, 135). ROS can also directly modify lipids to form lipid aldehydes, which oxidize protein lysine and cysteine residues, as well as DNA (131, 136). Interestingly, a group of transcription factors were shown to be susceptible to ROS modifications, making ROS an intracellular signaling messenger. For instance, NF-κB and AP1 can both be activated by ROS and increase expression of proinflammatory cytokines, which in turn further increases ROS production (137). This feed forward effect of ROS to inflammation indicate mutual causal relationship between oxidative stress and inflammation, which both lead to insulin resistance. However, whether oxidative stress or inflammation occurs first in the development of obesity is still largely debatable. Another transcription factor subject to ROS regulation is

hypoxia inducible factors (HIFs), HIF1 and HIF2 (138, 139). Under normal conditions, two conserved proline residues of HIF can by hydroxylated by prolyl hydroxylase domain protein 2 (PHD2) and targeted for pVHL-dependent proteasomal degradation (140). However, under hypoxic conditions with oxygen levels below 5%, PHD2 activity is inhibited and dimerization of HIF1α and HIF1β nucleus translocate and activate downstream gene expression, including the expression of IL1β (140). Studies over the years indicate mitochondrial ROS production under hypoxic condition is essential for HIF stabilization (140, 141).

Increased oxidative stress leads to oxidative damage of cellular components and cause insulin resistance. Therefore, ROS production has to be well controlled to preserve insulin sensitivity (115, 131). As a major location for ROS generation, a mitochondrion contains a number of antioxidant enzymes to eliminate ROS. As mentioned before, SODs detoxify superoxide to H_2O_2, and multiple enzymes are responsible for further detoxification of H_2O_2 (115). Peroxiredoxins (PRXs), glutathione peroxidases (GPXs), and catalase can all convert H_2O_2 to water and molecular oxygen (115). In addition to systems dedicated to elimination, mitochondrial ROS is also regulated at the production level. Major determinant of ROS generation include the redox status of the ETC itself and proton motive force (115, 142). Inhibitors of electron transport, which block the flow of electrons often lead to a more oxidized state of the ETC, increasing the capacity to generate ROS (125). Also, increased proton motive force leads to more

resistance of electron movement through the ETC, resulting in electron leakage and early reaction with oxygen (125). Both mitochondrial generated ROS and cytosolic ROS can oxidize redox sensitive cysteine residues (methionine residue can also be oxidized), within specific redox regulated proteins (143). However, this process can also be reversed by glutathione (2GSH/GSSG) and thioredoxin (Trx_{red}/Trx_{ox}). These two redox couples are highly abundant in both mitochondria and cytosol and both require their reducing ability from $NADPH/NADP^+$ (143). By allowing electrons to rapidly flow through these couples, the ratio of reduced versus oxidized glutathione and thioredoxin reflect the balance between reducing input ($NADPH/ NADP^+$) and oxidizing input (143). In summary, oxidative stress can be regulated at site of ROS generation, by enzyme activity of antioxidant enzymes, and the redox status of the protein targets.

Uncoupling protein 2 as a suppressor of ROS and inflammation

Uncoupling protein 2 (UCP2) belongs to a subfamily of mitochondrial anion-carrier proteins-uncoupling proteins (144, 145). The founding member of the subfamily-UCP1 is predominantly expressed in brown fat and browning white fat (also know as beige fat), involved in non-shivering thermogenesis by uncoupling oxidative phosphorylation and ATP production (146). UCP2 shares 60% of sequence homology with UCP1, but does not seem to be involved in adaptive thermogenesis (147). Expression of UCP2 has been shown leads to reduce mitochondria-derived reactive oxygen species (148-152). UCP2 has also been suggested to function as C4 metabolites transporter and regulate glucose and lipid metabolism (153). In humans, UCP2 polymorphism is associated with type 2 diabetes and atherosclerosis (154). The correlation mainly comes from a G/A single nucleotide polymorphism in the promoter region of *UCP2*. A-allele, which was associated with increased UCP2 expression, was linked to a reduced risk of obesity in Austrian, French Caucasians and Japanese populations (155-157). This same allele is also associated with reduced risk of diabetic neuropathy in Germany Caucasians and reduced risk of coronary artery disease in French Caucasians (158). However, in Austrian Caucasian patients, G allele was associated with lower blood triacylglycerol levels and higher insulin sensitivity (159). Interestingly, in mouse strains A and J, which have higher level of UCP2 expression are resistant to diet-induced obesity compared to C57BL/6J mice (160).

Studies based on various mouse models and cell lines indicate UCP2 is a major endogenous ROS suppressor. For instance, in UCP2 deficient mice, plasma total glutathione levels decreased about 40% compared to the matched wild type mice, indicating loss of overall antioxidant capacity is due to UCP2 deficiency (161). Additionally, increased aorta hydrogen peroxide level was observed in mice lacking UCP2 (161). In heart, expression of UCP2 is required for reducing mitochondrial superoxide level and protects heart from anoxia-reoxygenation damage (162). Additionally, macrophages that are deficient of UCP2 expression generate more ROS and have increased capacity to remove *T.gondii* tachyzoites infection *in vitro* (163). LPS treatment of wild type macrophages down-regulate UCP2 to increase mitochondrial ROS and activate MAPK (164, 165). Consistent with the important role of ROS in the activation of inflammatory pathways, UCP2 deficient macrophages produce more inflammatory cytokines (166). In summary, UCP2 serves as a master regulator of ROS production and inflammation in different cells and is a good candidate to improve insulin sensitivity in obese, diabetic patients.

Sirtuin3 as a suppressor of ROS and inflammation

Sirtuin3 (SIRT3) is a member of the sirtuin family, which is a class of NAD+ dependent protein deacetylases (167). Sirtuin family members each have distinct tissue distributions, subcellular localization, enzymatic activity, and recognize a different subset of targets (168). SIRT3 is primarily localized to the mitochondria, and highly expressed in tissues with high oxidative capacity, such as skeletal muscle, liver, and brown adipose tissue (169). Expression and activity of SIRT3 has been closely associated with development of obesity and type 2 diabetes (170). High fat diet feeding dramatically decreased SIRT3 expression in both skeletal muscle and liver, while caloric restriction increased its activity (170-172). High fat diet fed SIRT3 deficient mice develop severe obesity, dyslipidemia, hepatic inflammation, and insulin resistance compared to wild type control mice (173).

At the cellular level, SIRT3 is a master regulator of multiple pathways involved in energy metabolism. For instance, SIRTs regulate ATP production by directly regulating the acetylation status of a number of Complex I and II components (174, 175). SIRT3 can also regulate the TCA cycle or the urea cycle by deacetylating and activating glutamate dehydrogenase, or ornithine transcarbamoylase, respectively (176). By deacetylating long-chain acyl-CoA dehydrogenase (LCAD), SIRT3 can also promote β-oxidation (170). Importantly, SIRT3 as a mitochondria enzyme plays important roles in regulating

mitochondrial ROS levels. The function of SIRT3 as a ROS suppressor mainly comes from the deacetylase activity of SIRT3 on manganese superoxide dismutase (MnSOD) and isocitrate dehydrogenase 2 (IDH2) (175). As was previously discussed, MnSOD is a major mitochondrial antioxidant, while IDH2 is a TCA cycle enzyme and produces NADPH, which is required to restore the reduced pool of glutathione (177, 178). In SIRT3 deficient mice, there was a dramatic increase of oxidative stress in skeletal muscle and also increased JNK activation (179). Targeted knockdown of Sirt3 in myoblasts induced JNK activation and insulin resistance (179). In a high fat diet fed mouse model, SIRT3 knock out lead to increased expression of hepatic inflammatory cytokines, such as IFNγ, IL1β, TNFα, and IL6 (170). In summary, SIRT3 plays important roles in regulating both ROS generation and inflammation, making it a desirable therapeutic target to improve obesity and insulin resistance.

Current objectives

Obesity and obesity related syndrome such as cardiovascular disease, non-alcoholic fatty liver disease, and insulin resistance all root in metabolic dysregulation or ectopic accumulation of lipids. In adipose tissue, liver, heart, immune cells and any other cell type involved in lipid metabolism, fatty acids transport to storage in lipid droplets, oxidation in mitochondrial, or signaling in the nucleus and other organelles, all require fatty acid binding proteins (FABPs). FABPs make up a family of about 30kDa fatty acid trafficking proteins with 9 members expressed at different levels and exhibit different tissue distribution (180).

FABP4, which is also known as aP2 (adipocyte protein 2), is expressed predominantly in adipocytes and macrophages (181). Interestingly, a genetic polymorphism in the human FABP4 promoter region, which leads to its decreased expression, has been associated with decreased risk for type 2 diabetes and cardiovascular disease (182). About 20 years ago, Hotamisligil et al. showed that mice lacking FABP4/aP2 are protected from diet induced insulin resistance, despite similar weight gain compared to wild type mice (31). In the same paper, TNFα was directly implicated in the contribution to insulin resistance and loss of FABP4/aP2 decrease serum TNFα level (31). Follow-up studies demonstrated, FABP4/aP2 deficient mice were shown protected from the development of atherosclerosis, which seemed to solely rely on the loss of

FABP4/aP2 expression in macrophages (183). Studies over the years have indicated that FABP4/aP2 deficient macrophages are also protected from endoplasmic reticulum stress (ER stress) and inflammation. For instance, FABP4/aP2 deficient macrophages have reduced NF-κB activity, decreased inflammatory markers, such as cyclooxygenase 2 (COX2) and inducible nitric-oxide synthase (iNOS) (184). However, the mechanism, which explains how FABP4/aP2 deficiency leads to suppression of inflammation and ER stress, was not well defined (185). In my thesis, I will provide data to show that loss of FABP4/aP2 in macrophages leads to increased expression of both UCP2 and SIRT3. The expression of these two proteins both contributed to the suppressed inflammation of FABP4 deficient cells. Up regulation of UCP2 also contribute to reduced ER stress of FABP4 inhibition, while SIRT3 up regulation contribute to improved mitochondrial resistance to LPS induced dysfunction and improved β-oxidation in FABP4/aP2 deficient cells. Importantly, in a clinical surgery model, human bariatric surgery, the inverse relationship between FABP4 and UCP2 persists in the subcutaneous adipose tissue before and one week after surgery. This relationship may contribute to the insulin sensitizing effect of the surgery, an observation that has yet to be defined in the field.

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CHAPTER TWO

Uncoupling Lipid Metabolism from Inflammation Through FABP-dependent Expression of UCP2

Hongliang Xu, Ann V. Hertzel, Kaylee A. Steen, Qigui Wang, Jill Suttles and David A. Bernlohr (2015) *Molecular and Cellular Biology* 35(6):1055-65

Hongliang Xu performed experiments in their entirety from figures 1-9.

Summary

Chronic inflammation in obese adipose tissue is linked to endoplasmic reticulum (ER) stress and systemic insulin resistance. Targeted deletion of the murine adipocyte fatty acid binding protein (FABP4/aP2) uncouples obesity from inflammation, although the mechanism underlying this finding has remained enigmatic. Herein we show that inhibition or deletion of FABP4/aP2 in macrophages results in increased intracellular free fatty acids and elevated expression of uncoupling protein 2 (UCP2) without concomitant increases in UCP1 or UCP3. Silencing of UCP2 mRNA in FABP4/aP2 deficient macrophages negated the protective effect of FABP loss and increased ER stress in response to palmitate or lipopolysaccharide (LPS). Pharmacologic inhibition of FABP4/aP2 with the FABP inhibitor HTS01037 also up-regulated UCP2 and reduced expression of BiP, CHOP and XBP1s. Expression of native FABP4/aP2 (but not the non-fatty acid binding mutant, R126Q) into FABP4/aP2 null cells reduced UCP2 expression suggesting that the FABP-FFA equilibrium controls uncoupling protein 2 expression. FABP4/aP2 deficient macrophages exhibit UCP2-dependent reduction in intracellular reactive oxygen species, decreased mitochondrial protein carbonylation and are resistant to LPS-induced mitochondrial dysfunction. These data demonstrate that FABP4/aP2 directly regulates intracellular FFA levels and indirectly controls macrophage inflammation, and ER stress by regulating the expression of UCP2.

Introduction

Obesity-linked metabolic disorders including insulin resistance, fatty liver disease, and coronary arterial disease share the common signature of chronic inflammation and endoplasmic reticulum (ER) stress (1, 2). Macrophage and T cell infiltration and activation in adipose tissue plays a key role in affecting adipokine synthesis and secretion thereby regulating systemic insulin resistance (3). Inflammatory cytokines increase oxidative stress and decrease the protein-folding efficiency of the ER initiating a counter regulatory unfolded protein response (UPR) (4) involving pancreatic ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring enzyme 1 (IRE1). Such concomitant activation leads to the downstream activation of response pathways and the induction of inflammatory signaling networks via JNK (c-Jun N-terminal kinase) and/or NF-κB (nuclear factor kappa B) (1).

Lipid metabolism in macrophages has been shown to play an important role in triggering inflammation and ER stress (5, 6) and has led to the identification of critical proteins that regulate the obesity-metabolic disease axis. For example, genetic ablation of the adipocyte fatty acid binding protein (FABP4, also known as aP2) in macrophages alone is sufficient to protect the mice from development of atherosclerosis and dyslipidemia (7). FABP4/aP2 is a cytoplasmic fatty acid carrier protein that mediates intracellular fatty acid trafficking and a number of

hypotheses have been proposed for why the loss of FABP4/aP2 results in metabolic improvement (6). Moreover, small molecules that target FABP4/aP2 have been developed as potential therapeutics (8). However, conflicting reports exist concerning the effectiveness of these inhibitors using cell-based and animal models (9). FABP4/aP2 deficient macrophages exhibit suppressed inflammatory signaling, attenuated activation of the NF-κB pathway and decreased ER stress (6, 10). Consistent with a role for FABP4/aP2 as a key determinant in obesity-linked inflammation, genetic variation in the human FABP4/aP2 promoter that leads to decreased expression of the protein in adipose tissue is associated with lower serum triglyceride levels, reduced coronary disease and type 2 diabetes (11). The biochemical processes underlying the effects of FABP4/aP2 deficiency on macrophage lipid metabolism and ER stress and inflammatory pathways are not understood, but may be linked to the accumulation of intracellular unsaturated fatty acids, particularly palmitoleic acid (6, 10).

The investigation herein describes the novel finding that UCP2 is up regulated selectively in macrophages from FABP4/aP2 null mice and that increased expression of UCP2 plays an important and essential role in alleviating ER stress and decreasing inflammation (12, 13). Unlike its structural homolog UCP1 that is highly expressed in brown fat, UCP2 is more broadly expressed in various tissues and cells, functions as a sensor of mitochondrial oxidative stress and is generally considered to be cytoprotective (14). Moreover, unsaturated fatty acids

increase UCP2 expression in macrophage cells suggesting that the FABP – fatty acid equilibrium is central to mediating metabolic homeostasis.

Research Design and Methods

Cell lines. FABP4/aP2 knockout and wild type macrophage cells were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (FBS). Raw264.7 macrophages as well as UCP2 knockdown Raw264.7 macrophages were maintained in DMEM (Invitrogen) with 10% FBS. Peritoneal macrophages were isolated from C57Bl/6J animals. 1-2 $X10^6$ cells were plated and incubated overnight (15).

Intracellular fatty acid analysis. Monolayers of cells were washed with phosphate-buffered saline and harvested into 2 mL of 100 mM sodium acetate (pH 3.9). Lipids were extracted into hexane: isopropanol: H_2O (3:2:2) and centrifuged at 3000 rpm for 10 minutes to achieve phase separation. The aqueous phase was dried under nitrogen and lipids solubilized in 1 mL chloroform. Samples were loaded onto equilibrated HF Bond Elut NH2 column (Agilent Technology), washed with chloroform: isopropanol (2:1) to remove neutral lipids and fatty acids eluted with 2% acetic acid in diethyl ether. The fatty acid eluate was dried and resolubilized in isopropanol for measurement of fatty acid abundance (NEFA kit, Wako) or submitted to the Metabolomics Resources Core of the Mayo Clinic (Rochester MN) for fatty acid composition analysis. The fatty acid composition analysis was carried out by LC-MS with C17:0 spiked in each sample as an internal standard.

Isolation of stromal vascular cells. Epididymal fat pads were dissected from wild type C57Bl/6J and FABP4/aP2 KO mice (n=6) maintained on high fat diet for 12 weeks (16). Briefly, fat pads were minced and digested with Type I collagenase in Krebs-Ringers-Hepes buffer supplemented with 10 mg/ml BSA. After incubation at 37° C for 1 hour, the mixture was filtered with cell strainer (100μm Nylon, FALCON) to remove undigested tissues. The stromal vascular fraction was collected by centrifugation of the flow-through cells at 500 g for 10 minutes. The stromal vascular fraction was washed and TRIzol reagent used for RNA isolation. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

shRNA knockdown of UCP2 in macrophages. Raw264.7 and FABP4/aP2 KO macrophages were transduced with shRNA lentivirus as described previously (17). GFP scrambled and Ucp2 targeting sequences were obtained from Open Biosystems. Ucp2 (NM_011671) targeting sequence (UCP2 kd): 5'CCGGTCTCCCAATGTTGCCCGTAATCTCGAGATTACGGGCAA CATTGGGAGATTTTTG-3'; alternative UCP2 targeting sequence (UCP2-2 kd): CCGGCCCA GCCTACAGATGTGGTAACTCGAGTTACCACATCTGTAGGCTGGGTTTTTG-3'; the scrambled sequence: 5'-AACGTACGCGGAATACTTCGA-3'.

Expression analysis by Quantitative Real time PCR (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen), and reverse transcribed to cDNA using iScript according to the manufacturer's protocol (Bio-Rad). qRT-PCR amplification was performed on a Bio-Rad CFX 96 Real-Time System using SYBR Green Supermix (Bio-Rad). Transcription factor II E (TFIIE) was used as an internal control to normalize expression unless specified otherwise. Primer sequences are provided in Table 1.

Reverse transcription PCR analysis (RT-PCR). RT-PCR was used to identify UCP1, UCP2, and UCP3 expression in macrophages. Primers for UCP1 forward: GCCAGGCTTCCAGTACC
ATTA, UCP1 reverse: TGGTACGCTTGGGTACTGTCC; UCP2 forward: CCAGAGCACTGT
CGAAGCCT, UCP2 reverse: GCAGCCATTAGGGCTCTTTTG; UCP3 forward: AGAACCCA
GGGGCTCAGAG, UCP3 reverse: AAAACGGAGATTCCCGCAGTA.

Target	Forward primer	Reverse primer
UCP2	ACTGTGCCCTTACCATGCTCC	ATTGGTAGGCAGCCATTAGGG
PPARγ	GCCATTGAGTGCCGAGTC	TGTGGATCCGGCAGTTAAG
LxRα	TCAAGGGAGCACGCTATGTC	TTCCTCTTCTTGCCGCTTC
CD36	TGGAGCTGTTATTGGTGCAG	TGGGTTTTGCACATCAAAGA
Arginase	AACACGGCAGTGGCTTTAACC	GGTTTTCATCTGGCGCATTC
SCD1	CCTACGACAAGAACATTCAATCCC	CAGGAACTCAGAAGCCCAAAGC
iNOS	AGCGAGTTGTGGATTGTCC	TCTCTGCCTATCCGTCTCG
Catalase	CCAGCGACCAGATGAAGCAG	CCACTCTCTCAGGAATCCGC
Gpx4	GCTGTGCGCGCTCCAT	CCATGTGCCCGTCGATGT
SOD1	GCCAATGATGGAATGCTCTCC	CAATCTGACTGCTGGAAAGGAC
SOD2	CCGAGGAGAAGTACCACGAG	GCTTGATAGCCTCCAGCAAC
Prd3	TTA AACATGGTTAGTTGCTAGTACAAGGA	TTGAGACATGATCTAAGAATAGCCTCCTA
ALDH2	TTTATCCAGCCCACCGTGTTC	CAAGCCCATACTTAGAATCATTGG
XBP-1s	CTGAGTCCGAATCAGGTGCAG	GTCCATGGGAAGATGTTCTGG
XBP-1u	TGGCCGGGTCTGCTGAGTCCG	GTCCATGGGAAGATGTTCTGG
F4/80	TTTGGCTATGGGCTTCCAGTC	TCAGCAACCTCGTGTCCTTGAG
TFIIE	CAAGGCTTTAGGGGACCAGATAC	CATCCATTGACTCCACAGTGACAC

Table 1- Primers used for quantitative PCR to measure gene expression

Cellular respiratory Assay. Macrophage respiratory assay was performed on a XF24 (Seahorse Biosciences) (18). Macrophages were plated on V7 microplates at a density of 300,000 cells per plate, incubated overnight, and then cells were treated either with vehicle or lipopolysaccharide (LPS) (100ng/ml) for 6 hours. During the assay, cells were exposed to compounds in the following order: 2 μM oligomycin, 0.4 μM FCCP, and 4 μM antimycin A.

Hydrogen Peroxide (H_2O_2) assay. H_2O_2 quantification was determined using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to the manufacturer's protocol with modification. Briefly, cells were scraped into phosphate buffer (pH7.4) and inactivated at 95°C for 10 minutes. After spin down of cell debris, 50 μL of supernatant were loaded with 50 μL working solution. Following 30 minutes incubation, fluorescence was measured using a microplate reader with excitation at 540 nm and emission at 590 nm.

Membrane potential measurement. Mitochondrial proton motive force was measured by tetramethylrhodamine, methyl ester (TMRM) staining (Invitrogen). Briefly, cells were washed with PBS, and incubated in 1 mL of KRH buffer (pH7.4) with 20 nM final concentration of TMRM for 30 minutes. Then cells are washed with PBS and harvested into 300 μL KRH buffer. 150 μL of each sample

was load into 96-well plate and fluorescence was measured using microplate reader with excitation at 531 nm and emission at 572 nm.

Mitochondrial isolation. Cells were scraped into isolation buffer (20 mM Tris (pH 7.4), 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) supplemented with protease inhibitors and lysed with 20 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700*g* for 10 minutes to remove nuclei and unbroken cells. Mitochondria were pelleted by centrifugation at 12,000*g for 15 minutes*.

Immunoblotting. Cells were lysed in RIPA buffer supplemented with protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, membranes were incubated with primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to LI-COR IR Dye for 1 hour and visualized using LI-COR Odyssey infrared imaging (LI-COR biotechnologies). The antibodies used were anti-UCP2 (Santa Cruz Biotechnology, C-20), anti-HNE (Millipore), anti-DDIT3 (anti-CHOP) (Abcam), anti-GPR78 (Bip) (Santa Cruz Biotechnology, H-129), anti-Cox2 & anti-iNOS (BD Transduction Laboratories), anti-β-Actin (Sigma Aldrich) and anti-ATP synthase-α subunit (MitoSciences).

Cytokine TNFα measurement. Secreted TNFα in medium (8hr) was measured with Mouse TNF ELISA Set from BD Biosciences according to the manufacturer's instruction.

Fatty acid oxidation assay. Fatty acid oxidation was carried out as described by Wiczer and Bernlohr (19). Briefly, cells were incubated for 1 hour at 37°C in Krebs-Ringers-Hepes buffer (pH7.4), containing 5.4 mM glucose and 400 μM [^{14}C] palmitic acid bound to 100 μM fatty acid free BSA. Cells were scraped from the plates and transferred with media into 20 mL glass reaction vials containing a center reaction tube filled with 400 μL 1M sodium hydroxide. 70% perchloric acid was added to the media (final concentration of 7%), incubated for 1 hour with shaking at 80 rpm. After incubation, the content of the center tube was transferred into 10 mL liquid scintillation fluid and the $^{14}CO_2$ determined by liquid scintillation counting.

Statistical analysis. All data in the paper are expressed as standard deviation (± SD). Statistical significance was determined using an unpaired, two-tailed Student *t* test.

Results

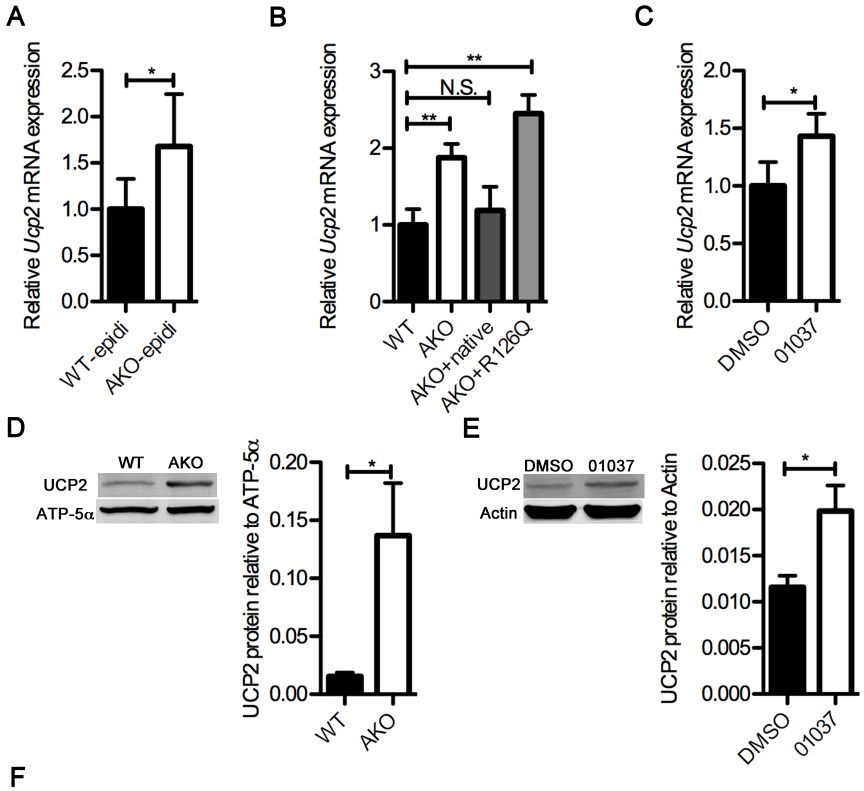
Loss or inhibition of FABP4/aP2 in macrophages leads to increased UCP2 expression. Previous reports have demonstrated that the intracellular fatty acid pool in FABP4/aP2 deficient macrophages and adipocytes is increased and has an altered composition (6, 20). Work in other systems, particularly in the liver, has shown that the expression of UCP2 mRNA and protein is increased with obesity, regulated by fatty acids and that lipid overload leads to increased expression of UCP2 as part of a counter regulatory cycle (14, 21). Since FABPs establish an intracellular equilibrium between bound and free fatty acids, we hypothesized that loss of FABP4/aP2 would result in increased availability of lipid and up-regulation of UCP2. To test this hypothesis, we evaluated the expression of uncoupling proteins using qRT-PCR and determined that UCP2 mRNA is increased approximately 60% in FABP4/aP2 deficient SVF cells compared to that of wild type mice (Fig. 1A). Similarly, UCP2 mRNA levels were increased ~ 2-fold in cell lines derived from FABP4/aP2 deficient mice (referred to as AKO macrophages) compared to control cells (Fig. 1B). The phenotype of FABP4/aP2 deficiency in macrophages can be mimicked by treatment of cultured macrophages with a previously identified and characterized chemical inhibitor of FABPs, HTS01037 (8). Treatment of Raw264.7 macrophages with HTS01037 also significantly increases UCP2 mRNA level (Fig. 1C). To further confirm that the up-regulation of UCP2 is responsive to FABP4/aP2 deficiency, AFABP/aP2$^{-/-}$ macrophages were reconstituted with either a wild type FABP4/aP2 or a non-fatty acid binding mutant of AFABP/aP2 (R126Q) (22) to a level comparable to

that of FABP4/aP2 in wild type macrophages (results not shown). Re-expression of wild type FABP4/aP2, but not the R126Q mutant, reduces UCP2 mRNA to a level comparable to that in wild type macrophages (Fig. 1B) implying that the intracellular FFA-FABP equilibrium is a major control element for UCP2 expression. Analysis of UCP2 protein levels in FABP4/aP2 deficient macrophages show that UCP2 is significantly increased compared to that of wild type (Fig. 1D). Moreover, the treatment of Raw264.7 macrophages with the FABP inhibitor HTS01037 also increases UCP2 protein level (Fig. 1E). Furthermore, the increased expression of uncoupling protein was specific for UCP2 as there was no evidence for any expression of UCP1 or UCP3 (Fig. 1F). These results in sum indicate that FABP4/aP2-FFA equilibrium controls the expression of the UCP2.

Unsaturated fatty acids induce UCP2 expression in macrophages via PPARγ. Several previous reports have shown that in multiple systems UCP2 expression can be induced by unsaturated fatty acids (14, 23-25). Moreover, FABPs are identified as lipid chaperones involved in establishment of the bound vs. free FFA equilibrium (16, 26). Consistent with previous work, intracellular free fatty acids in FABP4/aP2$^{-/-}$ macrophages were increased ~70% compared to that of wild type (Fig. 2A) (6). Moreover, fatty acid composition analysis revealed that monounsaturated fatty acid levels such as oleic acid (18:1), palmitoleic acid (16:1 *cis*) and palmitelaidic acid (16:1 *trans*) were selectively elevated in

Figure 1- Loss of FABP4/aP2 increases UCP2 expression.

(A) UCP2 mRNA level normalized to macrophage F4/80 in the stromal vascular fraction of epididymal adipose tissue obtained from high fat diet fed wild type (WT) and AFABP/aP2$^{-/-}$ (AKO) mice. (B) UCP2 mRNA level in wild type, AKO, AKO+native (AFABP/aP2$^{-/-}$ macrophages reconstituted with WT AFABP/aP2), AKO+R126Q (AFABP/aP2$^{-/-}$ macrophages reconstituted with a non-fatty acid binding mutant of AFABP/aP2). (C) UCP2 mRNA level in Raw264.7 macrophages treated with 30 μM HTS01037. (D) UCP2 protein expression in AKO and WT macrophage mitochondrial fraction determined by western blot. (E) UCP2 expression in Raw264.7 macrophages treated with 30 μM HTS01037 determined by western blot. (F) RT-PCR amplification of UCP1, 2 and 3 from wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages. Amplification of UCP1 from brown fat (B-fat) was used as positive control for UCP1 expression, while UCP3 expression control was amplified from a heart cDNA sample. (* $p<0.05$, ** $p<0.01$)


A
Relative Ucp2 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
WT-epidi
AKO-epidi
B
Relative Ucp2 mRNA expression
3
2
1
0
**
N.S.
**
WT
AKO
AKO+native
AKO+R126Q
C
Relative Ucp2 mRNA expression
2.0
1.5
1.0
0.5
0.0
*
DMSO
01037
D
WT
AKO
UCP2
ATP-5α
UCP2 protein relative to ATP-5α
0.20
0.15
0.10
0.05
0.00
*
WT
AKO
E
DMSO
01037
UCP2
Actin
UCP2 protein relative to Actin
0.025
0.020
0.015
0.010
0.005
0.000
*
DMSO
01037
F
Primer
Template
UCP1
UCP2
UCP3
B-fat
WT
AKO
WT
AKO
WT
AKO
Heart
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp

FABP4/aP2$^{-/-}$ macrophages (Fig. 2B). To test which molecular species of fatty acid could induce UCP2 expression in macrophages, Raw264.7 cells were treated for 24 hours with different fatty acids (4:1 FFA/bovine serum albumin) and the expression of UCP2 evaluated. Results demonstrate that polyunsaturated fatty acids, including DHA (22:6), EPA (20:5), linoleate (18:2) and monounsaturated fatty acid oleate (18:1), palmitoleate (16:1) can all induce UCP2 expression in macrophages, while the saturated fatty acid palmitate (16:0) was unable to elicit any response (Fig. 2C). Unsaturated fatty acids have been shown to act as ligands of a family of transcription factors - peroxisomal proliferator-activated receptors (PPARs) which are involved in regulating the expression of a cohort of genes involved in lipid metabolism (27). PPARγ is the major form of PPARs expressed in macrophages and suppresses the expression of a large set of inflammatory genes (28). qRT-PCR analysis shows that both PPARγ and its target genes such as liver X receptor alpha (LxRα), cluster of differentiation 36 (CD36), arginase and stearoyl-CoA desaturase-1 (SCD1) were up-regulated in FABP4/aP2$^{-/-}$ macrophages compared to that of wild type macrophages. On the contrary, the expression of the proinflammatory gene, inducible nitric oxide synthase (iNOS), is down regulated in FABP4/aP2$^{-/-}$ macrophages (Fig. 2D). In addition, treatment of wild type peritoneal macrophages with the FABP inhibitor HTS01037 shows similar results (Fig. 2E). In order to determine the role of PPARγ in macrophage UCP2 expression, Raw264.7 cells were treated with the PPARγ agonist troglitazone. Troglitazone

treatment increased the expression of UCP2 in macrophages, as well as LXRa (Fig. 2F). On the other hand, treatment of FABP4/aP2$^{-/-}$ macrophages with PPARγ antagonist GW9662 reduced UCP2 expression to a level comparable to that of wild type (Fig. 2G).

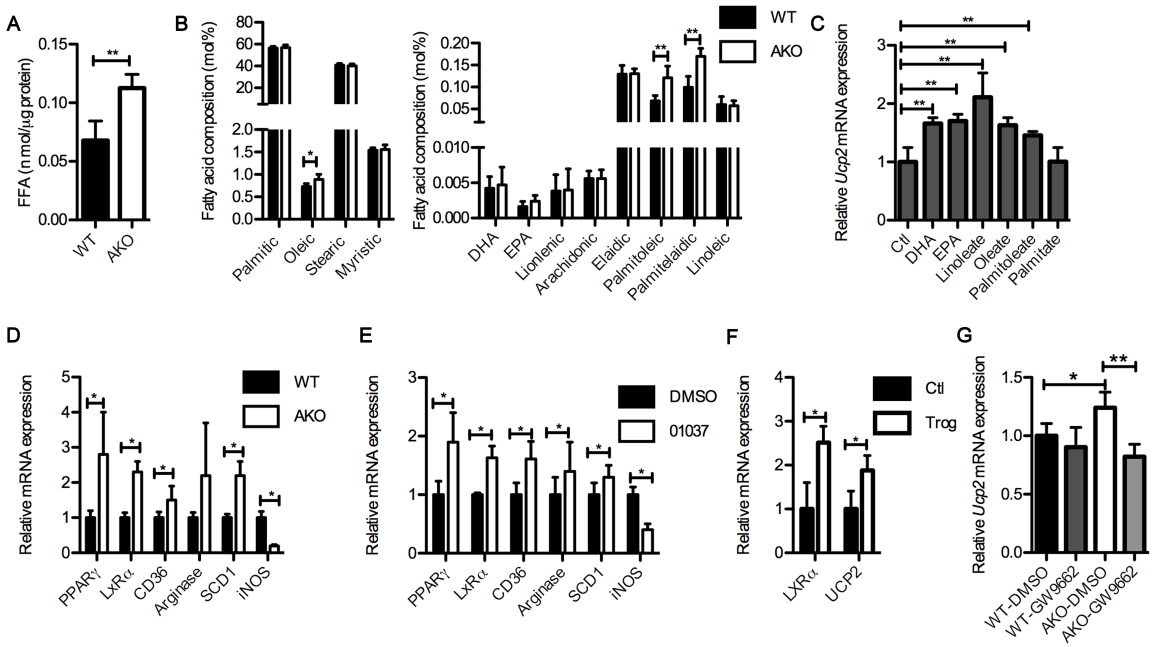
UCP2 up-regulation negates palmitate-induced ER stress in FABP4/aP2 deficient macrophages. The alleviation of lipid-induced macrophage ER stress can be accomplished either by knocking out FABP4/aP2 genetically or inhibiting the FABP-fatty acid interaction with small molecule inhibitors (6). In addition, the reduction in macrophage ER stress has been shown to offer protection against atherosclerosis (29). We therefore evaluated the role of UCP2 up-regulation in FABP4/aP2$^{-/-}$ macrophages as mediating reduced ER stress in palmitate-treated macrophages by silencing UCP2 in AKO and Raw264.7 macrophages (Fig. 3A and B). Spliced x-box binding protein 1 (XBP-1s) (30, 31) was markedly down-regulated in Raw264.7 cells treated with HTS01037, while the level of the unspliced form was increased, suggesting loss of FABP4/aP2 protects macrophages from ER stress (Fig. 4A). Treatment of UCP2 knockdown and control cells with HTS01037 shows that UCP2 silenced macrophages have a basal increase of XBP-1s and have a blunted response to HTS01037 (Fig. 4B).

Lipid loading, especially palmitate treatment, has been shown to induce expression of proteins involved in ER stress response, such as C/EBP

homologous protein (CHOP) and immunoglobulin heavy chain binding protein (Bip) (6). To assess the role of UCP2 induction in regulating ER stress response to palmitate, UCP2 knockdown and control cells were pretreated with HTS01037 or vehicle and palmitate-induced ER stress was evaluated. Expression of CHOP and Bip with palmitate treatment was markedly reduced in response to HTS01037 treatment in macrophages and this protection was negated in UCP2 knockdown macrophages (Fig. 4C-E). In order to further determine the role of UCP2 in mediating the ER stress response, UCP2 was knocked down in FABP4/aP2 deficient macrophages (AKO-UCP2 kd) and palmitate-induced ER stress evaluated. Importantly, CHOP and Bip expression were significantly induced upon palmitate treatment in both wild type and AKO-UCP2 kd macrophages, but not in FABP4/aP2$^{-/-}$ (AKO) macrophages (Fig. 4F and G). Taken together, these results strongly indicate that UCP2 expression in FABP4/aP2 deficient macrophages plays an important role in mediating the reduced lipid-induced ER stress.

Figure 2 - Unsaturated fatty acids induce UCP2 expression via PPARγ.

(A) Intracellular free fatty acids measured in WT (wild type) and AKO (FABP4/aP2$^{-/-}$) macrophages. (B) Fatty acid composition in WT and AKO macrophages. (C) UCP2 mRNA level in Raw264.7 macrophages treated with 300 μM docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleate, oleate, palmitoleate, or palmitate. Fatty acids were added in complex to bovine serum album at a molar ratio of 4:1. (D) mRNA levels of PPARγ, liver X receptor alpha (LxRα), cluster of differentiation 36 (CD36), arginase, stearoyl-CoA desaturase 1 (SCD1) and inducible nitric oxide (iNOS) in WT and AKO peritoneal macrophages. (E) PPARγ, LxRα, CD36, Arginase, SCD1 and iNOS mRNA level in 10 μM HTS01037 treated WT peritoneal macrophages. (F) mRNA level of LxRα and UCP2 in Raw264.7 macrophages treated with 5 μM troglitazone for 24 hours. (G) mRNA level of UCP2 in WT and AKO macrophages treated with 5 μM GW9662 for 24 hours. (* $p<0.05$, ** $p<0.01$)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WT
AKO
B
Fatty acid composition (md%)
Palmitic
Oleic
Stearic
Myristic
Fatty acid composition (mol%)
DHA
EPA
Lionlenic
Arachidonic
Elaidic
Palmitoleic
Palmitelaidic
Linoleic
C
Relative Ucp2 mRNA expression
Ctl
DHA
EPA
Linoleate
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Palmitoleate
Palmitate
D
Relative mRNA expression
PPARγ
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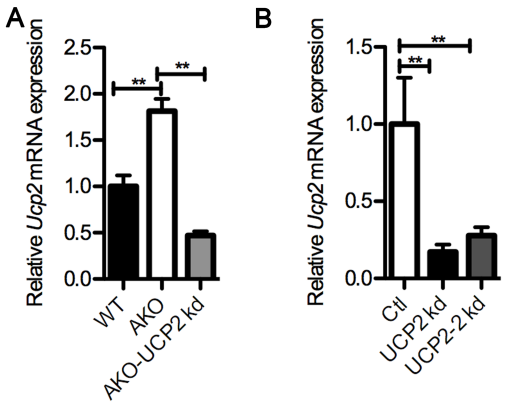
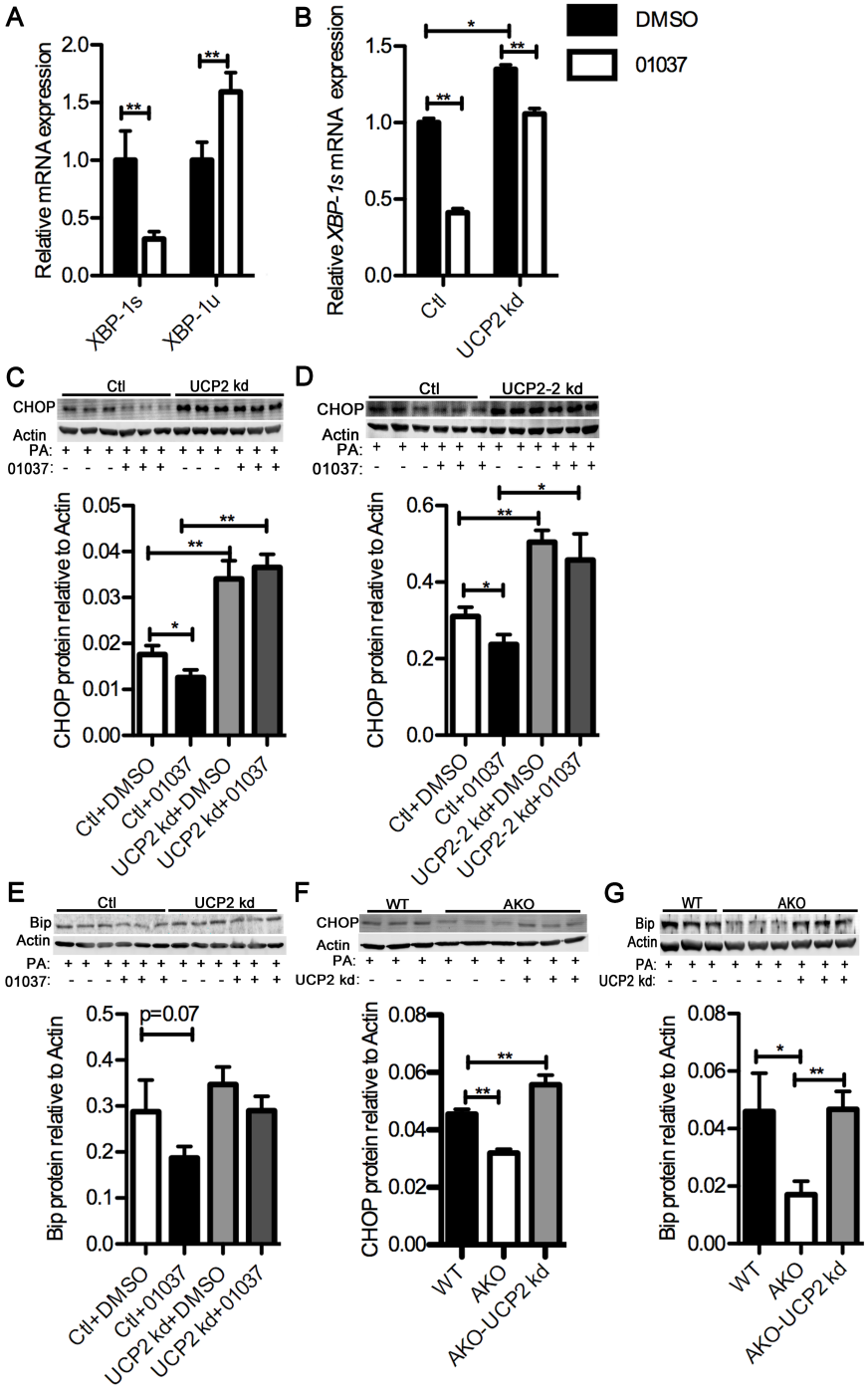


Figure 3 - Knock down of UCP2 in FABP4/aP2 deficient and Raw 264.7 macrophages.

(A) UCP2 mRNA levels in wild type (WT), AKO, and AKO-UCP2 kd (UCP2 knockdown AKO macrophages). (B) UCP2 mRNA levels in control cells, UCP2 kd and UCP2-2 kd (alternatively silenced UCP2 knock down cells) Raw264 macrophages. (* $p<0.05$, ** $p<0.01$)

Figure 4 - UCP2 up-regulation mediates decreased ER stress in FABP4/aP2 deficient macrophages.

(A) XBP-1s and XBP-1u mRNA levels in Raw264.7 macrophages treated with 30 μM HTS01037. (B) XBP-1s mRNA levels in UCP2 knockdown and control macrophage cell lines. (C-E) CHOP and Bip abundance determined by western blot in UCP2 knockdown and control cells pretreated with vehicle or HTS01037 (30μM) for 3 hours and treated with 500 μM palmitate for 16 hours. (F) CHOP abundance determined by western blot in WT, AKO and AKO-UCP2 kd (UCP2 knockdown AKO macrophages) cells treated with 300 μM palmitate for 24 hours. (G) Bip abundance determined by western blot in WT, AKO and AKO-UCP2 kd cells treated with 500μM palmitate for 12 hours. (* $p<0.05$, ** $p<0.01$)


A
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
**
**
XBP-1s
XBP-1u
B
DMSO
01037
Relative XBP-1s mRNA expression
1.5
1.0
0.5
0.0
*
**
**
Ctl
UCP2 kd
C
Ctl
UCP2 kd
CHOP
Actin
PA:
01037:
CHOP protein relative to Actin
0.05
0.04
0.03
0.02
0.01
0.00
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*
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
D
Ctl
UCP2-2 kd
CHOP
Actin
PA:
01037:
CHOP protein relative to Actin
0.6
0.4
0.2
0.0
*
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*
Ctl+DMSO
Ctl+01037
UCP2-2 kd+DMSO
UCP2-2 kd+01037
E
Ctl
UCP2 kd
Bip
Actin
PA:
01037:
Bip protein relative to Actin
0.5
0.4
0.3
0.2
0.1
0.0
p=0.07
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
F
WT
AKO
CHOP
Actin
PA:
UCP2 kd:
CHOP protein relative to Actin
0.08
0.06
0.04
0.02
0.00
**
**
WT
AKO
AKO-UCP2 kd
G
WT
AKO
Bip
Actin
PA:
UCP2 kd:
Bip protein relative to Actin
0.08
0.06
0.04
0.02
0.00
*
**
WT
AKO
AKO-UCP2 kd

UCP2 mediates the decreased inflammatory signaling in FABP4/aP2 deficient macrophages. FABP4/aP2$^{-/-}$ macrophages suppress the expression of inflammatory proteins such as cyclooxygenase-2 (Cox2) and iNOS (7, 10). In order to determine if UCP2 is involved in the suppression of inflammatory signaling in FABP4/aP2 knockout macrophages, wild type and FABP4/aP2$^{-/-}$ macrophages were treated with lipopolysaccharide (LPS) in the presence or absence of genipin, an UCP2 inhibitor (32) and inflammatory response was profiled. Consistent with the previous studies, LPS-induced Cox2 expression and TNF-α secretion were significantly lower in FABP4/aP2$^{-/-}$ macrophages (Fig. 5A and B). Moreover, LPS and genipin co-treatment of FABP4/aP2$^{-/-}$ macrophages significantly increased Cox2 expression and TNF-α secretion in FABP4/aP2$^{-/-}$ macrophages compared to that of LPS treatment alone (Fig. 5A and B) suggesting that increased UCP2 expression may attenuate inflammatory responsiveness. To further confirm UCP2's role in suppressing inflammatory signaling, control cells and UCP2 knockdown Raw264.7 macrophages were pretreated with HTS01037 and stimulated with LPS or LPS+INF-γ (interferon gamma). HTS01037 treatment significantly reduced LPS induced Cox2 and LPS plus IFN-γ induced iNOS expression in control cells (Fig. 5C-E). However, the effect of HTS01037 was significantly reduced, if not totally abolished, in UCP2 knockdown macrophages (Fig. 5C-E). Taken together, these data indicate that UCP2 plays an important role in mediating the reduced inflammation in

FABP4/aP2 deficient macrophages and that the key determinant of metabolic improvement in FABP deficient mice is up-regulation of UCP2.

UCP2 decreases the hydrogen peroxide level and oxidative stress in FABP4/aP2 deficient macrophages. A well-defined role for UCP2 is suppression of reactive oxygen species (ROS) production (13, 14). In order to determine the effect of FABP4/aP2 loss on ROS level in macrophages, the intracellular hydrogen peroxide levels in both FABP4/aP2$^{-/-}$ and wild type macrophages were determined. Figure 6A shows that FABP4/aP2$^{-/-}$ macrophages have significantly lower level of intracellular hydrogen peroxide. Moreover, treatment of control and FABP4/aP2$^{-/-}$ macrophages with genipin to inhibit UCP2 attenuated the decreased ROS levels in FABP deficient cells but had little effect on control macrophages (Fig. 6B). Interestingly, HTS01037 treatment of Raw264.7 macrophages, which mimics the knockdown of FABP4/aP2, also led to decreased intracellular hydrogen peroxide (Fig. 6C). Moreover, HTS01037 treatment of UCP2 knockdown cells was not able to reduce the intracellular hydrogen peroxide level (Fig. 6D) suggesting the effect of HTS01037 treatment on hydrogen peroxide level is likely to be mediated by UCP2 expression as well. Taken together, the results indicate that genetic loss or chemical inhibition of FABP4/aP2 leads to the reduced level of intracellular hydrogen peroxide in an UCP2-dependent manner.

Oxidative stress is a key contributor to mitochondrial dysfunction and apoptosis (33, 34). Consistent with decreased hydrogen peroxide that is indicative of reduced oxidative stress, most of the antioxidants, if not all, are decreased in FABP4/aP2 deficient macrophages (Fig. 6E). Surprisingly, silencing of UCP2 also leads to a decrease in the mRNA expression for several antioxidant enzymes (Fig. 6E and F). One of the effects of oxidative stress is protein carbonylation, the covalent modification of proteins with reactive lipid aldehydes that is linked to mitochondrial dysfunction (35). Utilizing an antibody directed to carbonylated proteins, and consistent with reduced ROS levels, FABP4/aP2$^{-/-}$ macrophages exhibit reduced protein carbonylation (Fig. 6G).

Figure 5 - UCP2 up-regulation mediates the decreased inflammation in FABP4/aP2 deficient macrophages.

(A) Cyclooxygenae 2 (Cox2) abundance measured by western blot in WT and AKO macrophages co-treated with or without LPS (100 ng/ml) ± genipin (40 μM) for 18 hours. (B) Secreted TNFα in cell culture medium determined by ELISA in WT and AKO macrophages co-treated with or without LPS (100 ng/ml) ± genipin (40 μM) for 8 hours. (C) Cox2 abundance determined by western blot in UCP2 knockdown and control macrophages pretreated with vehicle or HTS01037 (30 μM) for 3 hours and then treated with LPS (100 ng/ml) for 12 hours. (D and E) iNOS abundance determined by western blot in UCP2 knockdown and control macrophages pretreated with vehicle or HTS01037 (30μM) for 3 hours and then treated with LPS (100ng/ml) +IFN-γ (10U) for 12 hours (4 hours for UCP2-2 kd and control macrophages). (* $p<0.05$, ** $p<0.01$)

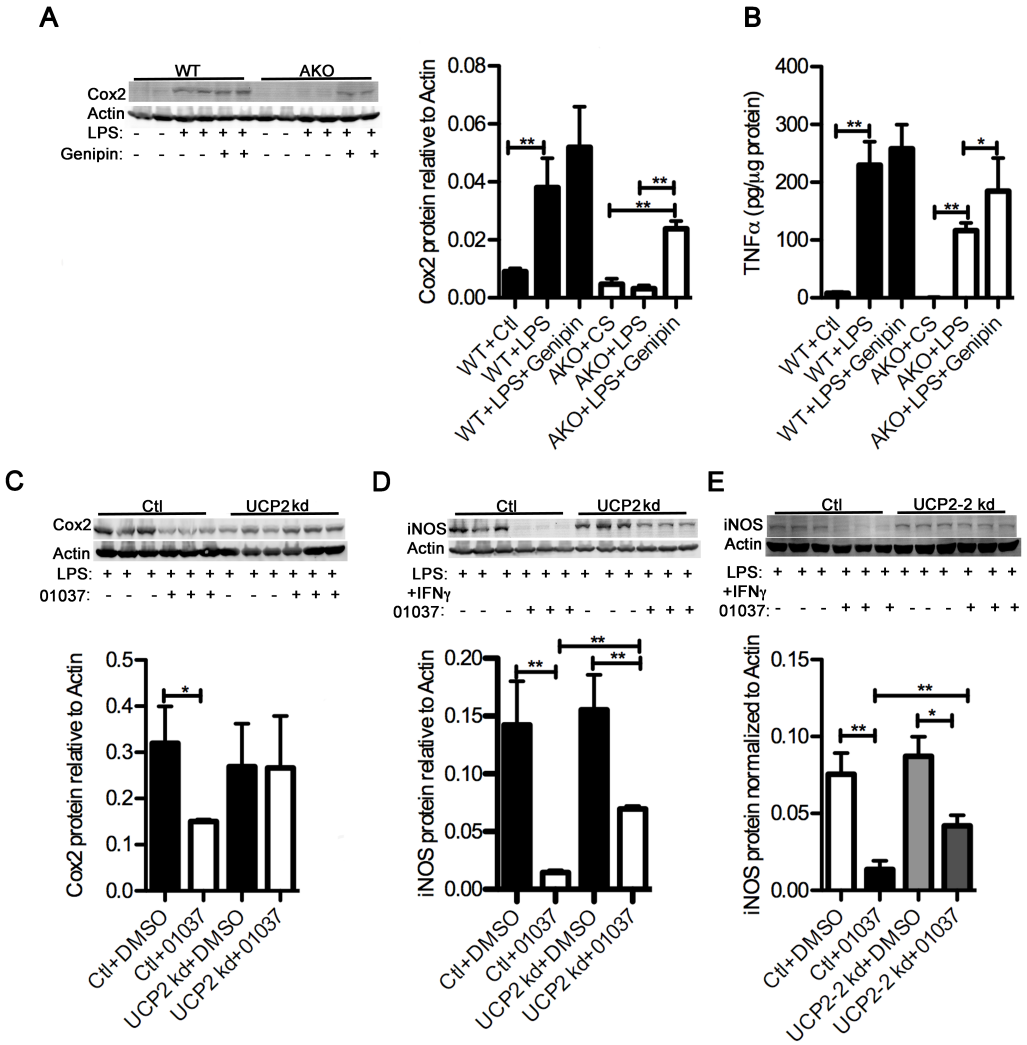
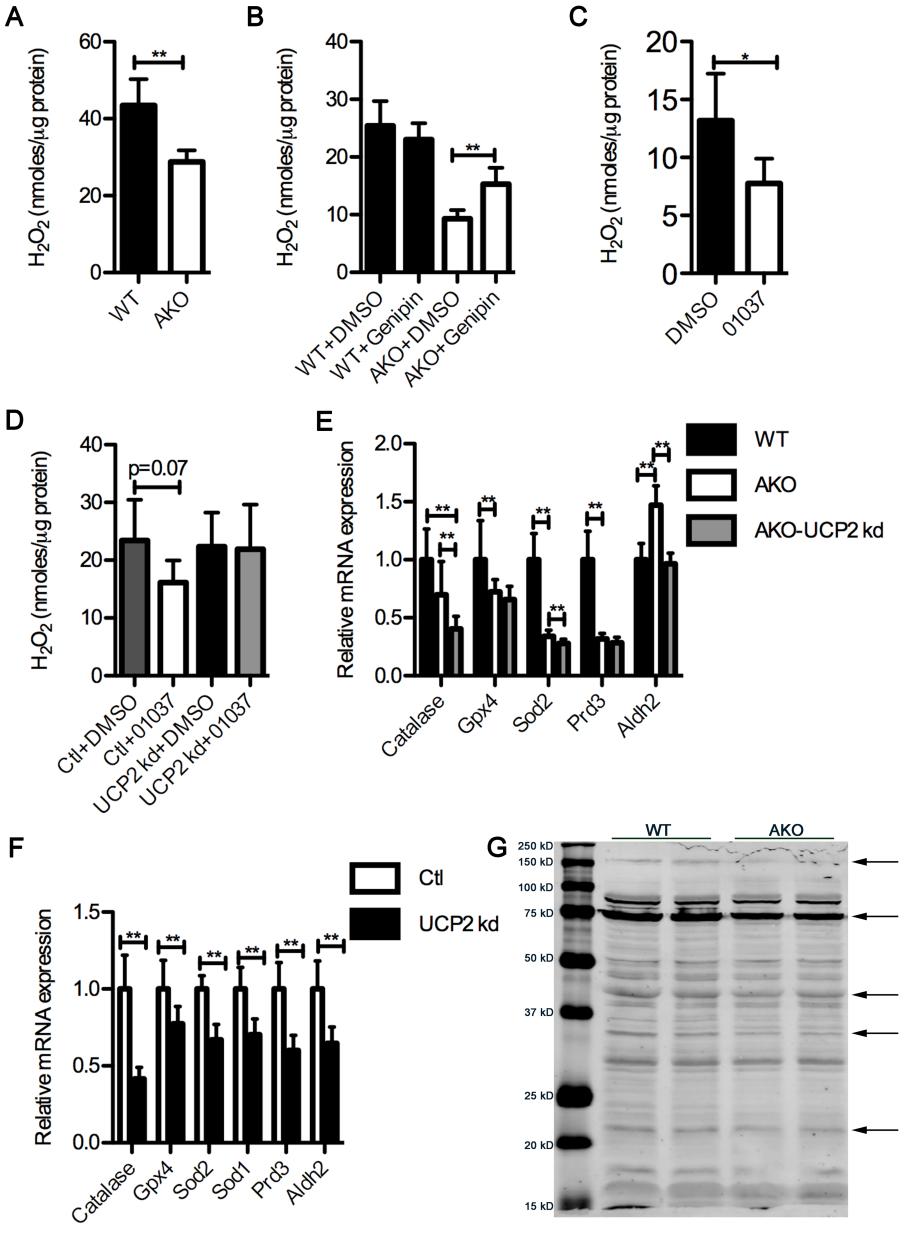

A
WT
AKO
Cox2
Actin
LPS:
Genipin:
Cox2 protein relative to Actin
0.08
0.06
0.04
0.02
0.00
WT+Ctl
WT+LPS
WT+LPS+Genipin
AKO+CS
AKO+LPS
AKO+LPS+Genipin
B
TNFα (pg/μg protein)
400
300
200
100
0
C
Ctl
UCP2 kd
Cox2
Actin
LPS:
01037:
Cox2 protein relative to Actin
0.5
0.4
0.3
0.2
0.1
0.0
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
D
iNOS
Actin
LPS:
+IFNγ
01037:
iNOS protein relative to Actin
0.20
0.15
0.10
0.05
0.00
E
UCP2-2 kd
iNOS protein normalized to Actin
UCP2-2 kd+DMSO
UCP2-2 kd+01037

Figure 6 - **UCP2 up-regulation decreases intracellular hydrogen peroxide in FABP4/aP2 deficient macrophages.**

(A) Intracellular hydrogen peroxide level in WT and AKO macrophages measured by Amplex Red Assay. (B) Intracellular hydrogen peroxide level in WT and AKO macrophages treated with vehicle or genipin (50 μM) for 6 hours. (C) Intracellular hydrogen peroxide level in Raw264.7 macrophages treated with HTS01037 (30 μM) for 24 hrs. (D) Intracellular hydrogen peroxide level in UCP2 knockdown and control cells treated with HTS01037 (30 μM) for 6 hours. (E) Antioxidant mRNA levels in WT, AKO, AKO-UCP2 kd cells. (F) Antioxidant mRNA levels in UCP2 knockdown and control cells. (G) Protein carbonylation measured by western blot with anti-HNE antibody in WT and AKO mitochondrial protein. (* $p<0.05$, ** $p<0.01$)


A
B
C
D
E
F
G
H_2O_2 (nmoles/μg protein)
WT
AKO
WT+DMSO
WT+Genipin
AKO+DMSO
AKO+Genipin
DMSO
01037
p=0.07
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
Relative mRNA expression
AKO-UCP2 kd
Catalase
Gpx4
Sod2
Prd3
Aldh2
Ctl
UCP2 kd
Sod1
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100 kD
75 kD
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25 kD
20 kD
15 kD

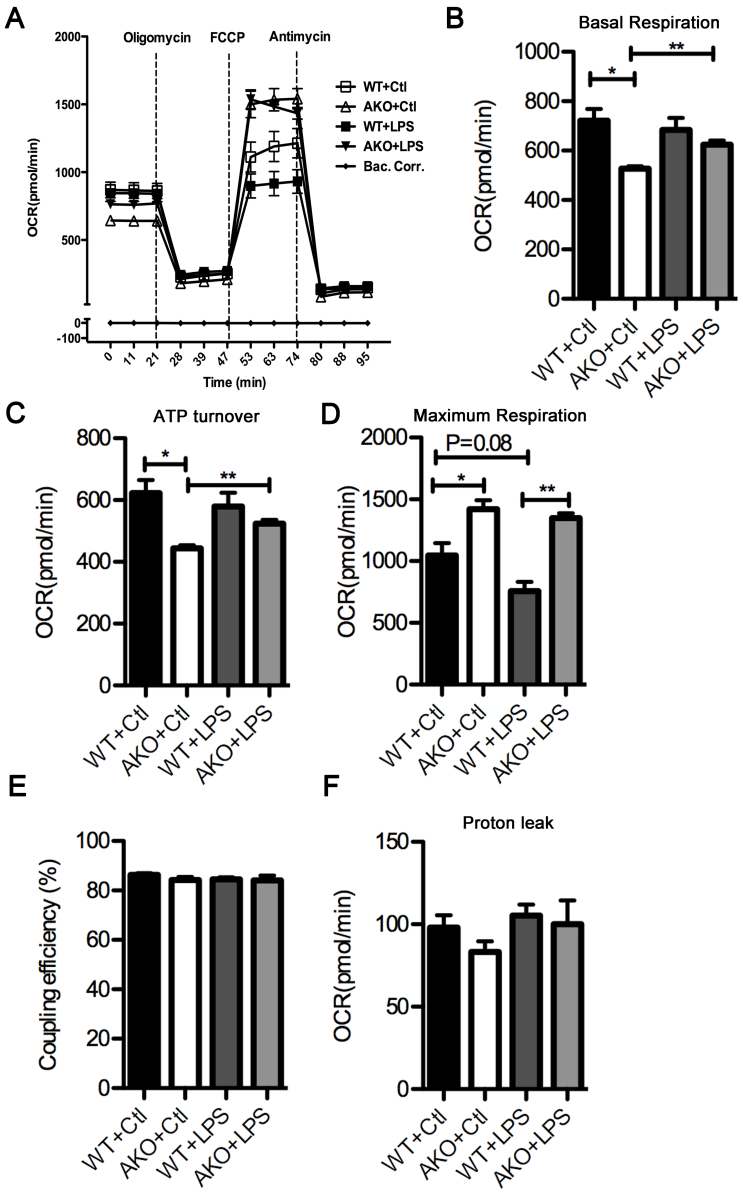


Figure 7 – Cellular respiration of FABP4/aP2 deficient and wild type macrophages.

(A-F) Oxygen consumption rate in WT and AKO macrophages treated with or without LPS (100ng/ml) for 4 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM). (* $p<0.05$, ** $p<0.01$)

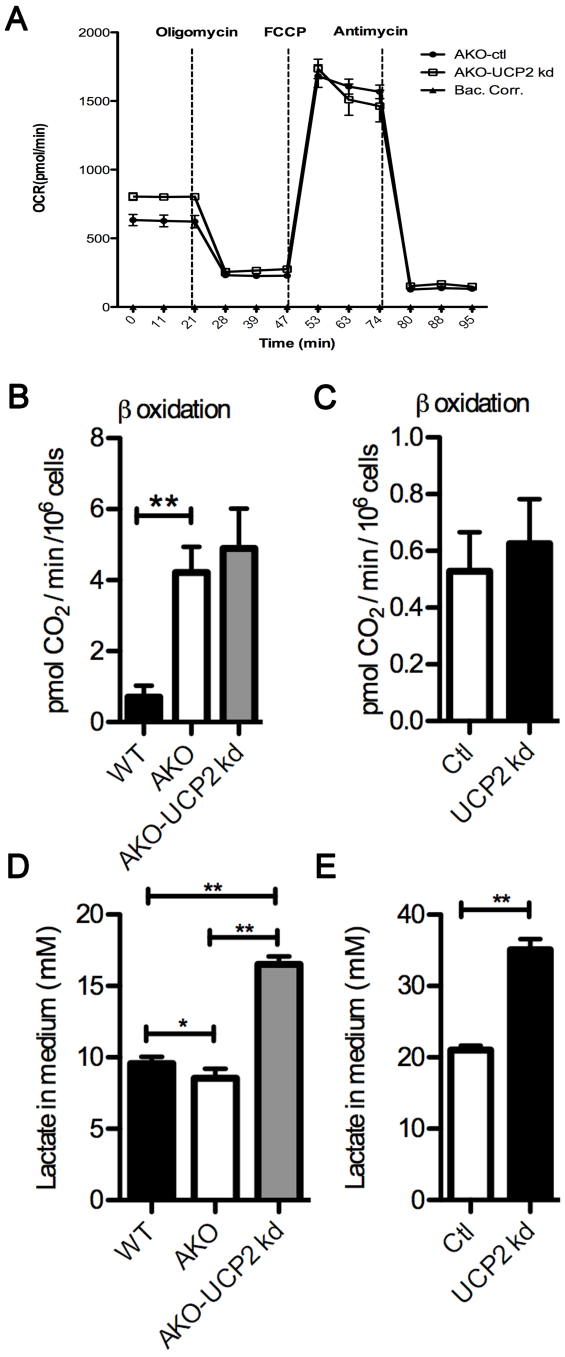
FABP4/aP2$^{-/-}$ macrophages have higher mitochondrial respiration capacity. Reduced carbonylation is predictive of improved mitochondrial function and to test this hypothesis, cellular respiration was evaluated both at basal level and in response to a LPS challenge. As shown in Figure 7, FABP4/aP2$^{-/-}$ macrophages exhibited a lower level of basal respiration, and ATP turnover, but a significantly higher level of maximum respiration. Upon LPS treatment, FABP4/aP2$^{-/-}$ macrophages have a significant increase of basal respiration and ATP turnover, while wild type macrophages lose the maximum respiration capacity (Fig. 7B-D). However, no difference of coupling efficiency and proton leak between the two cell lines or treatment was observed (Fig. 7E and F).

UCP2 mediates decreased basal respiration and lactate production of FABP4/aP2$^{-/-}$ macrophages but not the increased fatty acid oxidation. Consistent with a role of UCP2 in control of basal respiration, silencing of UCP2 in FABP4/aP2$^{-/-}$ macrophages increased basal, but not maximal respiration compared to control cells (Fig. 8A). Additionally, fatty acid oxidation was increased in macrophages lacking FABP4/aP2 (Fig. 8B). However, no difference of fatty acid oxidation was observed in UCP2 knock down FABP/aP2$^{-/-}$ or Raw264.7 macrophages (Fig. 8B and C) suggesting a UCP2-independent process. FABP4/aP2$^{-/-}$ macrophage cell culture medium had reduced lactate compared to wild type macrophages (Fig. 8D). This is consistent with the lower

basal respiration of FABP4/aP2$^{-/-}$ macrophages (Fig. 7B). In support of the role of UCP2 mediating the decreased lactate production in FABP4/aP2$^{-/-}$ macrophages, the lactate level was increased about 70% in the cell culture medium of UCP2 knock down FABP4/aP2$^{-/-}$ cells and UCP2 knockdown Raw264.7 cells compared with control macrophages (Fig. 8D and E).

Figure 8 - Metabolic impact of UCP2 knock down on macrophages.

(A) Oxygen consumption rate in FABP4/aP2 deficient and AKO-UCP2 kd macrophages. (B) β-oxidation measured in WT, AKO, AKO-UCP2 kd cells. (C) β-oxidation measured in UCP2 kd cells and control cells. (D) Cell culture media lactate level measured in WT, AKO, AKO-UCP2 kd cells. (E) Cell culture media lactate level measured in UCP2 knockdown and control cells. (* $p<0.05$, ** $p<0.01$)


A
Oligomycin
FCCP
Antimycin
AKO-ctl
AKO-UCP2 kd
Bac. Corr.
OCR(pmol/min)
Time (min)
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pmol CO2 / min / 10^6 cells
WT
AKO
AKO-UCP2 kd
C
β oxidation
pmol CO2 / min / 10^6 cells
Ctl
UCP2 kd
D
Lactate in medium (mM)
WT
AKO
AKO-UCP2 kd
E
Lactate in medium (mM)
Ctl
UCP2 kd

Discussion

Chronic activation of ER stress and inflammation in macrophages contributes to the pathogenesis of various metabolic disarrangements such as atherosclerosis and type 2 diabetes (4, 29). The stressors that can lead to macrophage ER stress and inflammation include oxidative stress, high level of intracellular cholesterol and saturated fatty acids (29). Prolonged elevation of macrophage ER stress and inflammation has been proposed to contribute to macrophage apoptosis and lead to plaque necrosis and rupture (2, 36). Therefore, understanding of biological processes involved in counteracting macrophage ER stress and inflammation is crucial for development of specific strategies to improve metabolic poise. Interestingly, FABP4/aP2 has been shown to play an important role in mediating both ER stress and inflammation in macrophages (6, 37). Genetic ablation or chemical inhibition of FABP4/aP2 alleviates macrophage inflammation and ER stress (6, 8, 10). The increase of monounsaturated fatty acids, PPARγ, and LxRα activity have all been suggested as mediating the anti-inflammatory and anti-ER stress effects of FABP4/aP2 deficiency (6, 10). However, the molecular relationship of FABP deficiency to inflammation and ER stress outcome is still unclear. Increased expression of UCP2 in FABP4/aP2$^{-/-}$ macrophages provides a mechanistic basis for the anti-inflammatory, anti-ER stress outcomes. Studies in a number of systems have implicated UCP2 as a major control element in macrophage ER stress, inflammation and diet induced atherosclerosis (38-40). Interestingly, a UCP2 promoter region -866G>A polymorphism which decreases UCP2 expression has been associated with

increased risk of obesity, decreased insulin level and type 2 diabetes (14). Moreover, type 2 diabetes patients bearing the G allele have a higher inflammatory status (41). In a second study, a UCP2 -86G>A polymorphism is associated with multiple chronic inflammatory diseases, including Crohn's disease, ulcerative colitis, and psoriasis (42).

It has been proposed that unsaturated fatty acids (or their metabolites) are potential ligands for LxRα and PPARγ (27). Fatty acids and the expression of LxRα and PPARγ, are increased in FABP4/aP2 deficient macrophages and have all been previously shown to up-regulate UCP2 expression (14). Interestingly, a previous study by Erbay et al. showed that the unsaturated fatty acid pool is increased in FABP4/aP2$^{-/-}$ macrophages (6). Results herein show that monounsaturated fatty acids are increased in FABP4/aP2$^{-/-}$ macrophages, and unsaturated, but not saturated, fatty acids induce UCP2 expression in macrophages, consistent with a report on fatty acid induction of UCP2 in liver cells (43). Moreover, given the increase in the intracellular free fatty acid pool in FABP4/aP2$^{-/-}$ macrophages, it is very likely they directly or indirectly activate PPARγ, thereby inducing UCP2 expression. However, this does not exclude the possibility that fatty acids may also directly regulate UCP2 activity.

Paradoxically, despite the existence of the other FABP in macrophages (FABP5/mal1), the deficiency of FABP4 leads to an anti-inflammatory and anti-

atherosclerotic phenotype (7). One explanation is that FABP4 has a higher affinity for unsaturated fatty acids compared to FABP5 (44). Therefore, the loss of FABP4 will affect the pool of available free unsaturated fatty acids, especially monounsaturated fatty acids. An additional possibility is the pools of fatty acids bound to FABP4 are distinct from those bound to FABP5, such that loss of FABP4 could exhibit a unique phenotype. Experiments to test these hypotheses are currently underway.

The reduced ER stress and inflammation demonstrated in both FABP4/aP2 deficient and FABP4/aP2 inhibited macrophages was markedly abolished or compromised by knockdown or inhibition of UCP2. This observation places UCP2 as a modulator between the FABP-FFA equilibrium and ER stress and inflammation, which is a role for UCP2 that has previously not been appreciated. It is noteworthy that the findings do not rule out contributions from other parallel pathways that reduce ER stress and inflammation. However, the results imply that the UCP2 mediated pathway is likely the major determinant of the FABP4/aP2 deficient macrophage phenotype since UCP2 knockdown or inhibition greatly compromised the protection from loss of FABP4/aP2 function.

The prominent and well-defined role of UCP2 is to suppress ROS (13). The data herein show that FABP4/aP2$^{-/-}$ macrophages have a lower level of intracellular hydrogen peroxide that is dependent on UCP2 expression as evidenced by

restoration via genipin inhibition of UCP2. Hydrogen peroxide has been shown to directly oxidize the side chains of several amino acids, particularly cysteine, thereby affecting the cellular redoxome. Alternatively, if not detoxified by antioxidants such as catalase, peroxiredoxin, or glutathione peroxidase, hydrogen peroxide could react with iron to form hydroxyl radicals and oxidize lipids. 4-hydroxynonenal (4-HNE) and 4-oxononenal (4-ONE) are the two most well studied lipid peroxidation products and have been shown to covalently modify proteins, a process termed as protein carbonylation. This frequently leads to loss or alteration of protein activity and mitochondrial dysfunction. (35, 45). Interestingly, a decreased level of mitochondrial protein carbonylation was observed in FABP4/aP2$^{-/-}$ macrophages consistent with improved mitochondrial function (35). The results in Figure 7 demonstrating that FABP4/aP2$^{-/-}$ macrophages were protected from LPS-induced loss of mitochondrial respiration capacity support this concept. Interestingly, it has been shown that LPS induced mitochondrial dysfunction mainly relies on the increased ROS production and suppression of ROS not only protects cells from LPS induced mitochondrial dysfunction but also greatly attenuates LPS induced inflammatory responses (46, 47). Further work is still required to determine if the decreased hydrogen peroxide level in FABP4/aP2$^{-/-}$ macrophages is responsible for the protection from LPS induced mitochondrial dysfunction.

Both hydrogen peroxide and oxidized lipids have also been shown to activate ER stress and inflammatory pathways (48, 49). It is tempting to speculate that the UCP2 suppression of ROS in FABP4/aP2$^{-/-}$ macrophages broadly impacts not only mitochondrial function but also endoplasmic reticulum function and inflammatory pathway activity (Fig. 9). Interestingly, knockdown of UCP2 in macrophages increases lactate production. The increased lactate production indicates increased energy production through glycolysis, a common indicator of electron transport chain dysfunction (50). Additionally, this may also explain UCP2 dependency of the lower basal respiration of FABP4/aP2 deficient macrophages. As a suppressor of ROS production, higher UCP2 expression in FABP4/aP2$^{-/-}$ macrophages leads to lower cellular oxidative stress. Since oxidative stress drives the upregulation of antioxidants, it is reasonable that most antioxidants are expressed at lower levels in FABP4/aP2$^{-/-}$ cells compared to wild type macrophages (Fig. 6E). The reduced UCP2 expression in macrophages, which leads to increased oxidative stress, would be predicted to lead to increased antioxidant protein expression. Paradoxically, in the FABP4/aP2$^{-/-}$, UCP2 kd macrophages, expression of antioxidant enzymes are down-regulated. This suggests UCP2 is involved in a more complex regulation of antioxidant expression. Nevertheless, the loss of antioxidant capacity of UCP2 knock down is consistent with a previous report showing that UCP2 deficient mice have reduced antioxidant capacity (38).

Overall, the studies herein provide a mechanistic basis for the metabolic improvement in FABP4/aP2 deficient cells generated by either genetic or pharmacologic means. Loss of FABP-FFA equilibrium is likely to increase the bioavailability of fatty acids, particularly unsaturated lipids that have the potential to increase the expression of UCP2. Whether this mechanism exists in non-macrophage cells and would pertain to the metabolic changes observed in FABP1, FABP2 or FABP3 null mice remains unknown (51). However, it may be that FABP-dependent modulation of intracellular FFA levels and therefore UCP2 expression may be a common property in many cell types.

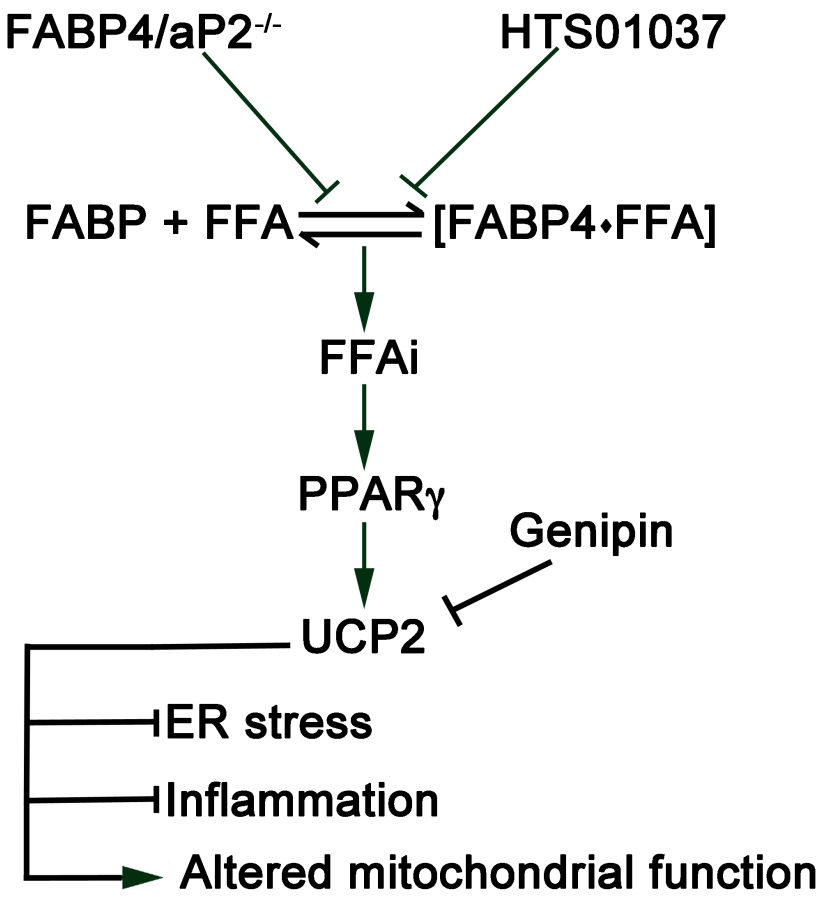

Figure 9 - Schematic model of the role of UCP2 up-regulation in macrophages.

Loss or inhibition of FABP4/aP2 increases the intracellular free fatty acid levels and induces expression of UCP2 via a PPAR-γ mediated pathway. Increased expression of UCP2 reduces oxidative stress in the mitochondrion, alleviates ER stress, inflammation and mitochondrial dysfunction in macrophages. FFAi refers to intracellular free fatty acids.

Acknowledgement

We would like to thank the members of the Bernlohr laboratory for helpful discussions during the study and preparation of the manuscript. We would also like to thank Dr. Edward McFalls, VA Medical Center, Minneapolis MN for helpful discussions and reagents in the preliminary phase of the study as well as Nicholas Kvalheim for assistance with real-time PCR measurements. The support of the Minnesota Supercomputer Institute is gratefully acknowledged. Supported by NIH R01 DK053189 to DAB, NIH T32 AG029796 to KAS, and the Minnesota Obesity Center (NIH P30 DK050456).

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CHAPTER THREE

Loss of Fatty Acid Binding Protein 4/aP2 Reduces Macrophage Inflammation Through Activation of SIRT3

Hongliang Xu, Ann V. Hertzel, Kaylee A. Steen and

David A. Bernlohr (2016) *Molecular and Cellular Biology* 35(6):055-65

This chapter contains an original research article previously published.

Hongliang Xu performed experiments in their entirety from figures 1-5.

Summary

Activation of pro-inflammatory macrophages plays an important role in the pathogenesis of insulin resistance, type 2 diabetes and atherosclerosis. Prior work using high-fat fed mice has shown that ablation of the adipocyte fatty acid binding protein (FABP4/aP2) in macrophages leads to an anti-inflammatory state both in situ and in vivo and the mechanism is linked, in part, to increased intracellular monounsaturated fatty acids and the up-regulation of UCP2. Herein, we show that loss of FABP4/aP2 in macrophages additionally induces SIRT3 expression and that monounsaturated fatty acids (C16:1, C18:1) lead to increased SIRT3 protein expression. Increased expression of SirT3 in FABP4/aP2 null macrophages occurs at the protein level with no change in SirT3 mRNA. When compared to controls, silencing of SIRT3 in Raw246.7 macrophages leads to increased expression of inflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (Cox2). In contrast, loss of SIRT3 in FABP4/aP2 deficient macrophages attenuates the suppressed inflammatory signaling, reduced reactive oxygen (ROS) production, LPS induced mitochondrial dysfunction and increased fatty acid oxidation. These results suggest that the anti-inflammatory phenotype of FABP4/aP2 null mice is mediated by increased intracellular monounsaturated fatty acids leading to the increased expression of both UCP2 and SirT3.

Introduction

The prevalence of syndromes associated with obesity, including insulin resistance, hypertension and dyslipidemia have increased over the last decade (1,2). High saturated fat or "western" diets and lack of exercise contribute to the epidemic of the metabolic syndrome (1,3,4). At the molecular level, multiple pathways are involved in the pathogenesis of metabolic diseases including lipotoxicity and chronic inflammation in multiple tissues such as liver, muscle, and adipose (5-8). Importantly, the infiltration and activation of immune cells such as T cells and macrophages play an essential role in the development of insulin resistance in adipose tissue (9,10). Of these activated immune cells, macrophages play an integral role in the production of inflammatory cytokines as well as the development of oxidative stress in adipose tissue (8).

Macrophage lipid metabolism is critical in mediating adipose tissue inflammation and oxidative stress (11,12). Work from our laboratory and many others have shown that the adipocyte fatty acid binding protein (FABP4, also know as aP2) plays an important role in the activation of macrophage inflammation (13-16). Ablation of FABP4/aP2 (AKO) in macrophages alone is sufficient to protect mice from diet induced atherosclerosis and dyslipidemia (14,16). FABP4/aP2 is a small 15 kDa lipid chaperone involved in intracellular fatty acid trafficking but recently has been shown to be secreted into the extracellular environment (17,18). Deficiency of FABP4/aP2 leads to suppressed inflammation, decreased

ER stress and decreased NF-κB activation in macrophages (13,19,20). Consistent with its role in ameliorating metabolic disorder in mice, a genetic variant at the promoter region of human FABP4/aP2, which leads to decreased expression, has been associated with reduced risk of coronary disease and type 2 diabetes (21).

Previous work has shown that the monounsaturated fatty acids palmitoleate and oleate are specifically increased in FABP4/aP2 deficient macrophages and are linked to the selective up-regulation of uncoupling protein 2 (UCP2) (22). Increased expression of UCP2 in macrophages attenuates oxidative stress and reduces inflammatory signaling. However, increased UCP2 expression alone is not sufficient to explain the increased fatty acid oxidation and resistance from lipopolysaccharide (LPS) induced mitochondrial dysfunction measured in FABP4/aP2 deficient cells suggesting additional, unappreciated regulatory mechanisms. Herein we report that SIRT3, a member of the sirtuin family, is specifically up-regulated in macrophages deficient in FABP4/aP2 and that monounsaturated fatty acids can induce SIRT3 up-regulation at the protein level. The resulting increase of SIRT3 expression in macrophages is linked to suppressed inflammatory signaling as well as decreased reactive oxygen production and increased fatty acid oxidation in FABP4/aP2 deficient macrophages. Furthermore, SIRT3 up-regulation protects FABP4/aP2 deficient macrophages from LPS induced mitochondrial dysfunction.

Research Design and Methods

Cell culture. Raw264.7 macrophages and Sirt3 knockdown Raw264.7 macrophages were maintained in DMEM (Invitrogen) with 10% fetal bovine serum (FBS). FABP4/aP2 knockout (AKO), wild type and Sirt3 knock down FABP4/aP2 deficient macrophages were maintained in RPMI 1640 (Invitrogen) with 5% FBS.

Quantitative RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA synthesis was performed by using iScript according to the manufacturer's protocol (Bio-Rad). qRT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix (Bio-Rad). Transcription factor II E (TFIIE) was used as an internal control to normalize expression. Primer sequences are provided in Table 1.

Silencing of Sirt3 in macrophages. Raw264.7 macrophages were transduced with a short hairpin RNA (shRNA) lentivirus targeting either Green Fluorescent Protein (GFP) or SirT3 as described previously (23). Green fluorescent protein and Sirt3 (GenBank accession number NP_001120823) targeting sequences were obtained from Open Biosystems: Sirt3 (kd-1), 5′-
CCGGGCCATCTTTGAACTTGGCTTTCTCGA
GAAAGCCAAGTTCAAAGATGGCTTTTT

G-3'; GFP, 5′-AACGTACGCGGAATACTTCGA-3′. Because SIRT3 is highly expressed in FABP4/aP2$^{-/-}$ macrophages (AKO) compared to wild type cells, two rounds of lentivirus infection was required to obtain significant silencing. To that end, FABP4/aP2$^{-/-}$ macrophages were first transduced with kd-1 followed by an additional shRNA lentivirus (kd-2), 5′-
CCGGCCTACTCCATATGGCTGACTTCTCGAGAAGTCAGCCA
TATGGAGTAGGTTTTT
G-3′.

Stromal vascular fraction isolation. Isolation of stromal vascular fraction was performed as described in Xu et al. (22). Briefly, epididymal fat pads were dissected from wild type and FABP4/aP2 knockout (AKO) mice (15 week old male C57BL/6J mice maintained on high saturated fat diet (BioServe F3282) for 12 weeks), minced and digested in Krebs-Ringers-HEPES (KRH) buffer supplemented with type I collagenase (Worthington) and bovine serum albumin (BSA) for one hour at 37° C. The mixture was filtered with 100-μm-pore-size nylon cell strainer (Falcon) to remove undigested tissues. The stromal vascular fraction (SVF) was collected by centrifugation at 500 × *g* for 10 min. After washing with KRH buffer, the SVF was either resuspended in TRIzol reagent for RNA isolation or in cell lysis buffer supplemented with protease inhibitors for protein assays. All experimental procedures using animals were reviewed and

approved by the University of Minnesota Institutional Animal Care and Use Committee.

Mitochondrial isolation and β-oxidation. Mitochondrial isolation were carried out as described in Xu et al. (22). Briefly, cells were scraped into ice-cold mitochondrial isolation buffer (20 mM Tris pH 7.4, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) and supplemented with protease inhibitors. Cells were then lysed with 20 strokes of a Dounce homogenizer and homogenates centrifuged at 700 × *g* for 10 min to remove nuclei and unbroken cells. Mitochondria were pelleted by centrifugation at 10,000 × *g* for 15 min at 4° C. Fatty acid oxidation was carried out as described previously (22).

Measurement of ROS. ROS production was measured by incubating cells with cell permeable 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen). Briefly, cells were washed with PBS, and incubated in 1 mL of KRH buffer (pH 7.4) with 10 μM final concentration of H_2DCFDA for 30 minutes. Then cells were washed with PBS and harvested into 300 μL KRH buffer. Of each sample, 150 μL was loaded into a 96-well plate and fluorescence was measured using a microplate reader with excitation at 488 nm and emission at 535 nm.

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')
Sirt3	GCTGCTTCTGCGGCTCTATAC	GAAGGACCTTCGACAGACCGT
TNFα	AGCCGATGGGTTGTACCTTGTCTA	TGAGATAGCAAATCGGCTGAGGGT
Mcp1	TCACCTGCTGCTACTCATTCACCA	TACAGCTTCTTTGGGACACCTGCT
Mmp9	CCCACATTTGACGTCCAGAGAAGAA	GTTTTTGATGCTATTGCTGAGATCCA
TFIIE	CAAGGCTTTAGGGGACCAGATAC	CATCCATTGACTCCACAGTGACAC

Supplementary table 1- Primers used for quantitative PCR to measure gene expression

Immunoblot analysis. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Calbiochem). 50 µg of protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with Odyssey blocking buffer (Li-Cor Biosciences), membranes were incubated with primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 h and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were anti-SIRT3 (Cell Signaling), anti-FABP4, anti-cyclooxygenase-2 (anti-Cox2) (BD Transduction Laboratories), anti-inducible nitric oxide synthase (anti-iNOS) (BD Transduction Laboratories), anti-superoxide dismutase (anti-SOD2) (Cell Signaling), anti-long-chain acyl-CoA dehydrogenase (anti-LCAD) (Abcam), anti-acetylated lysine (Cell Signaling), anti-β-actin (Sigma-Aldrich), and anti-ATP synthase-α subunit (MitoSciences).

Immunoprecipitation. Cells were washed twice with cold phosphate-buffered saline (PBS) and scraped into 1 ml of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, and 1% TritonX-100) supplemented with protease inhibitors and incubated for 10 minutes at 4° C. After centrifugation at 13,500 rpm for 10 minutes, supernatants were transferred to a new tube and the protein concentration was determined. 800 µg of protein lysates were pre-cleared by incubation with 30 µL protein G beads for 4 hours. After centrifugation,

supernatants were transferred to a new tube and 30 µL of a protein G bead slurry was added to each tube together with primary antibody or anti-rabbit IgG control antibody. After rotating overnight at 4° C, the pelleted beads were washed five times with 500 µL lysis buffer and resuspended in RIPA buffer. Precipitated proteins were released from the IgG beads by boiling the samples for 10 minutes and loaded onto a SDS-PAGE gel for separation.

Cellular respiratory assay. Macrophage respiratory assay was performed on a XF24 Analyzer (Seahorse Biosciences). Macrophages were plated on V7 microplates at a density of 200,000 cells per well a day prior to the assay. On the day of the experiment, cells were treated either with vehicle or lipopolysaccharide (LPS) (100 ng/ml) for 6 hours. The cells were then washed and incubated with assay media. During the assay, cells were exposed to compounds in the following order: 2 µM oligomycin, 0.4 µM FCCP, and 4 µM antimycin A.

Statistical analysis. All data in the paper are expressed as standard error of the mean (± SEM). Statistical significance was determined using an unpaired two-tailed Student T-test.

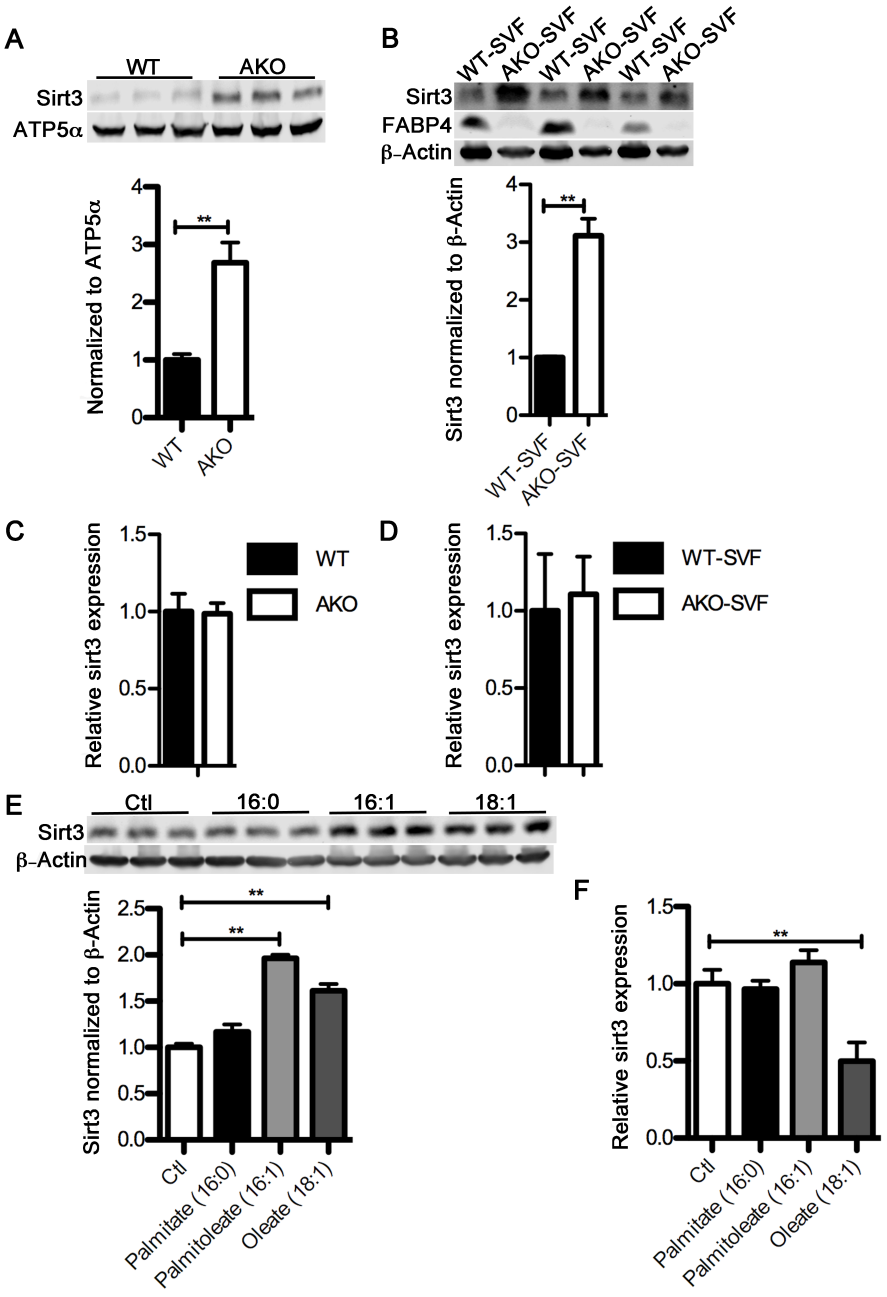
Results

Loss of FABP4/aP2 in macrophages increases SIRT3 expression. Previous work by Xu et al. (22) as well as Erbay et al. and Coe et al. (20, 24) has shown that loss of FABP4/aP2 alters the intracellular level and composition of fatty acids in both macrophages and adipocytes resulting in the accumulation of monounsaturated fatty acids, particularly C16:1 and C18:1. Interestingly, consumption of monounsaturated fatty acids, which are a major component of the Mediterranean diet, are associated with improved insulin sensitivity and decreased inflammation in both humans and mice (25). In muscle, oleic acid treatment can activate the SIRT1-PGC1α (peroxisome proliferator-activated receptor γ coactivator 1-α) axis to increase fatty acid oxidation (26). More recently, Liu et al. reported that in monocytes, SIRT1 activation leads to an increase of both expression and activation of SIRT3, thus restoring immunometabolic homeostasis (27). Therefore, we hypothesized that loss of FABP4/aP2 will increase SIRT3 expression via an increased level of monounsaturated fatty acids. To test this hypothesis, we evaluated the protein level of SIRT3 in both wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages derived from wild type and FABP4/aP2$^{-/-}$ mice, respectively. SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages was more than doubled compared to that in wild type (Figure 1A). To further confirm that the loss of FABP4/aP2 leads to up-regulation of SIRT3 in macrophages, we isolated the stromal vascular fraction (SVF) from visceral adipose tissue of male high fat diet (HFD) fed WT and AKO mice. The

level of SIRT3 was increased about three fold in AKO-SVF compared to that of wild type (Figure 1B). Interestingly, the level of Sirt3 mRNA did not change between wild type and FABP4/aP2$^{-/-}$ cell lines or SVF fractions indicating the difference of SIRT3 expression came from regulation at the translational or post-translational level (Figure 1C and D). To explore the potential role of monounsaturated fatty acids on SIRT3 expression, Raw264.7 macrophages were treated with either MUFAs (palmitoleate and oleate) or palmitate. The results showed that MUFA treatment could induce SIRT3 expression in macrophages but not the saturated fatty acid palmitate (Figure 1E). Similar to the results in experimental mice, the increase in protein was not accompanied by an increase in SirT3 mRNA (Figure 1F). Taken together, these results suggest that the increased monounsaturated fatty acids, due to the genetic ablation of FABP4/aP2, may be responsible for the increased SIRT3 expression in macrophages.

Figure 1 - Loss of FABP4/aP2 increases SIRT3 expression.

(A) SIRT3 protein level normalized to ATP5α in the mitochondrial fraction of wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages. (B) SIRT3 protein level normalized to β-actin in the stromal vascular fraction of epididymal adipose tissue obtained from high fat diet (HFD) fed WT and FABP4/aP2 (AKO) null mice. (C) SIRT3 mRNA level in WT and AKO macrophages. (D) SIRT3 mRNA level in the stromal vascular fraction of epididymal adipose tissue obtained from HFD fed WT and AKO mice. (E) SIRT3 protein expression in Raw264.7 macrophages treated with 300 μM palmitate, palmitoleate, or oleate for 36 hours. (F) SIRT3 mRNA level in Raw264.7 macrophages treated with 300 μM palmitate, palmitoleate, or oleate for 36 hours. Fatty acids were added in complex to bovine serum albumin (BSA) at a molar ratio of 4:1(FFA/BSA). (*$p<0.05$, **$p<0.01$, n=3-6 per group)


A
WT
AKO
Sirt3
ATP5α
Normalized to ATP5α
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WT
AKO
B
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AKO-SVF
WT-SVF
AKO-SVF
WT-SVF
AKO-SVF
Sirt3
FABP4
β-Actin
Sirt3 normalized to β-Actin
**
WT-SVF
AKO-SVF
C
Relative sirt3 expression
WT
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D
Relative sirt3 expression
WT-SVF
AKO-SVF
E
Ctl
16:0
16:1
18:1
Sirt3
β-Actin
Sirt3 normalized to β-Actin
**
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Ctl
Palmitate (16:0)
Palmitoleate (16:1)
Oleate (18:1)
F
Relative sirt3 expression
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Ctl
Palmitate (16:0)
Palmitoleate (16:1)
Oleate (18:1)

SIRT3 expression is anti-inflammatory in macrophages. Diet induced obesity is accompanied by decreased SIRT3 expression in multiple tissues including liver, skeletal muscle, heart, and pancreas (28-32). Both genetic knock out animal models and clinical studies of type 2 diabetics have shown the important role of SIRT3 in maintaining insulin sensitivity in various tissues and organs (29,30,32). However, the role of SIRT3 in tissue macrophages has not been well defined, having been studied primarily in a sepsis model (27). To illustrate the relationship between SIRT3 expression and macrophage inflammation, we silenced Sirt3 in Raw264.7 macrophages by infecting cells with lentivirus encoding a SirT3 targeted shRNA. The knockdown cell line expressed about 50% of SIRT3 protein compared to the control cells (Figure 2A). Co-treatment of Sirt3 silenced macrophage cells with LPS and interferon γ (IFNγ) induced a significantly higher level of iNOS expression in Sirt3 knockdown Raw264.7 macrophages compared to control cells (Figure 2B). In addition, macrophages treated with palmitate or palmitoleate, which have been shown to prime macrophages towards a pro- or anti-inflammatory state, respectively, regulated inflammatory marker genes. Decreased expression of SIRT3 led to increased palmitate induced Cox2 expression, and compromised the ability of palmitoleate to reduce Cox2 expression (Figure 2C). Additionally, Sirt3 knockdown Raw264.7 macrophages expressed higher transcript levels of the inflammatory cytokines TNFα and MCP1 (Figure 2D). In summary, loss of SIRT3 in macrophages results in an elevated inflammatory state.

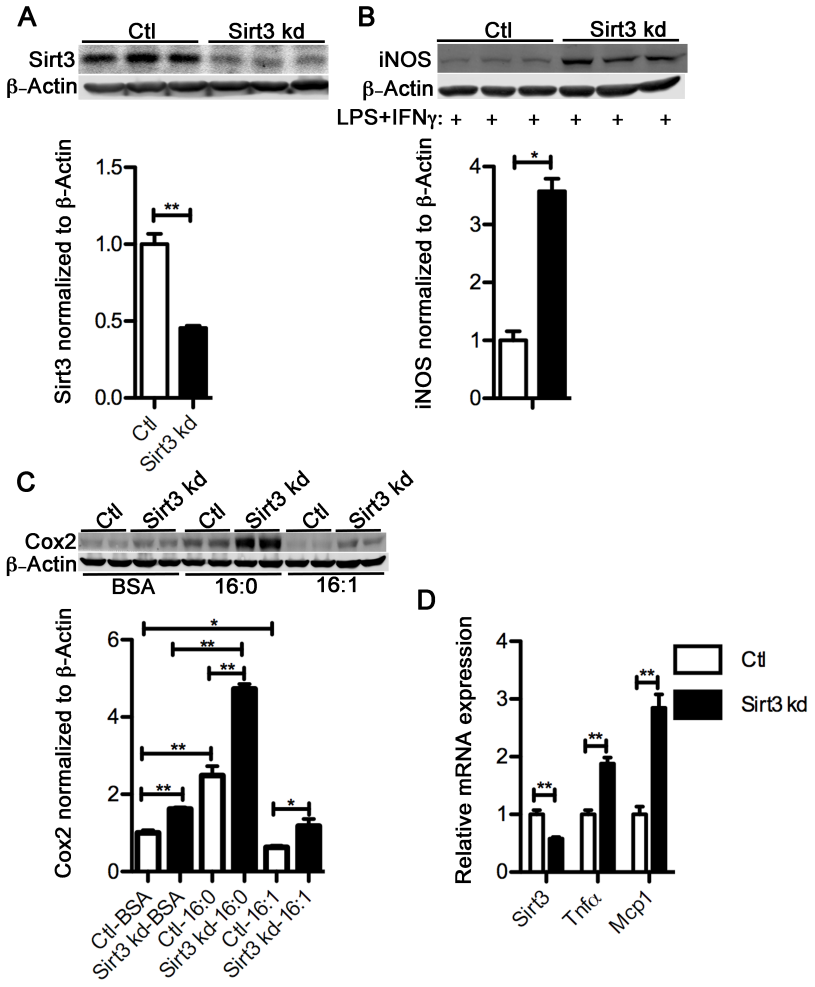


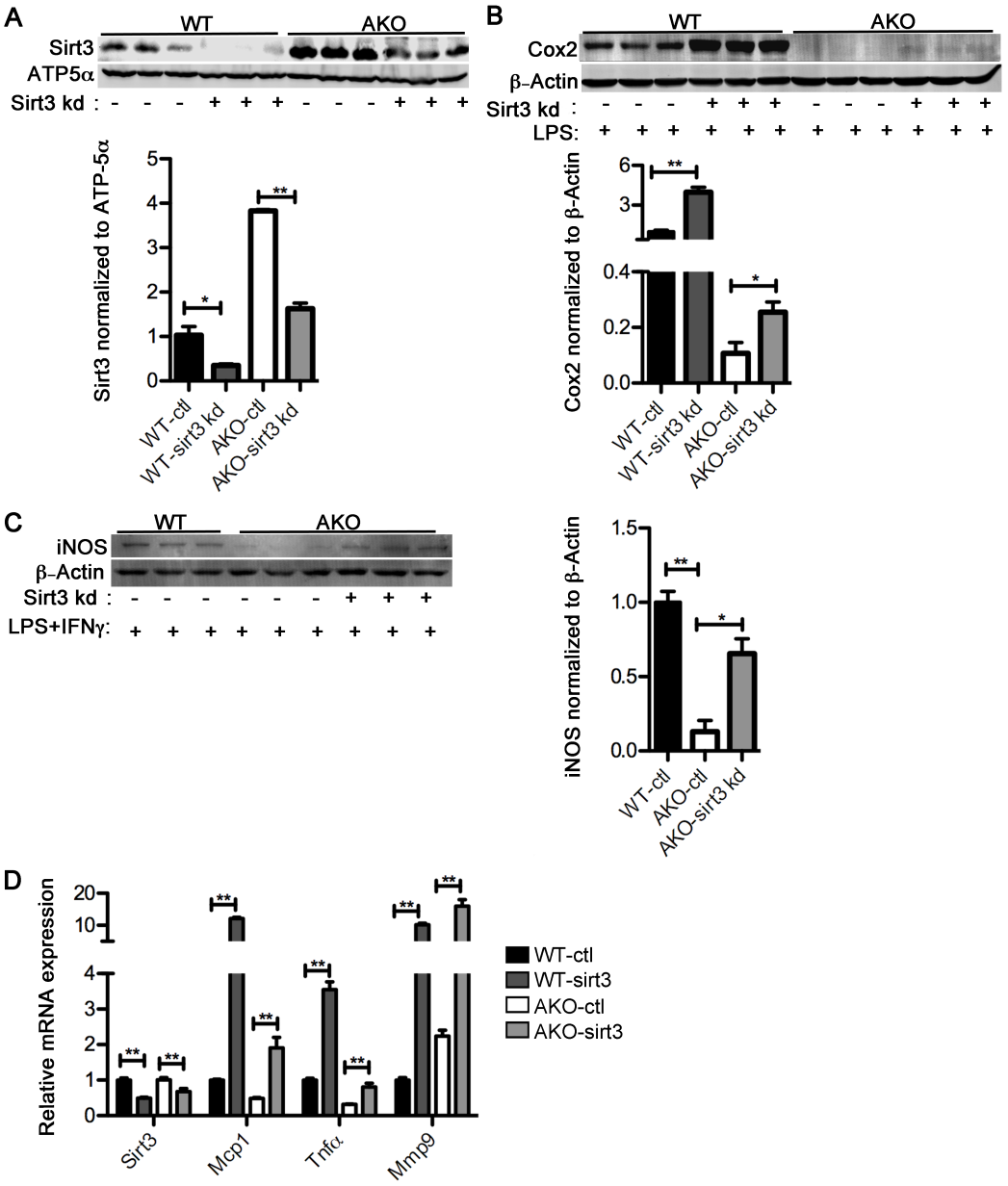
Figure 2 - Loss of SIRT3 is proinflammatory in macrophages.

(A) SIRT3 expression in SIRT3 knockdown Raw264.7 macrophages (Sirt3 kd) and control knockdown cells (Ctl). (B) iNOS abundance measured by western blot in Ctl and Sirt3 kd macrophages co-treated with LPS (100 ng/ml) and IFNγ (10U) for 14 hours. (C) Cox2 abundance in palmitate or palmitoleate (300 μM) (4:1 fatty acids to BSA) treated Ctl and Sirt3 kd macrophages. (D) mRNA levels of Sirt3, Tnfα and Mcp1 in Ctl and Sirt3 kd macrophages. (*p<0.05, **p<0.01, n=3 per group)

SIRT3 mediates the decreased inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages. Loss of FABP4/aP2 reduces macrophage inflammatory markers such as Cox2 and iNOS (13). In order to determine if SIRT3 plays a role in the suppressed inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages, we silenced SIRT3 (sirt3kd) in both wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) backgrounds (Figure 3A). LPS treatment induced higher expression of Cox2 in both WT-sirt3kd and AKO-sirt3kd macrophages compared to their corresponding control cell lines. The basal expression of SIRT3 was similar between wild type and AKO-sirt3 kd macrophages, however the expression of Cox2 was still much lower in AKO-sirt3kd (Figure 3B). Therefore, SIRT3 expression in AKO is partially responsible for the reduced inflammatory signaling. Similarly, knockdown of Sirt3 in FABP4/aP2$^{-/-}$ cells dramatically increased LPS and IFNγ induced iNOS expression (Figure 3C). Additionally, genetic knockdown of Sirt3 increased the basal expression of the inflammatory cytokines, MCP1, TNFα, and MMP9 mRNA in WT and FABP4/aP2$^{-/-}$ cells compared to their respective controls (Figure 3D). In conclusion, increased SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages is at least partially responsible for the reduced inflammatory signaling.

Figure 3 - SIRT3 up regulation mediates the decreased inflammation in FABP4/aP2$^{-/-}$ macrophages.

(A) SIRT3 protein expression in the mitochondrial fraction of WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages. (B) Cox2 abundance determined by western blot in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd cells treated with LPS (100 ng/ml) for 16 hours. (C) iNOS abundance determined by western blot in WT-ctl, AKO-ctl and AKO-sirt3 kd cells co-treated with LPS+IFNγ for 18 hours. (D) mRNA level of SIRT3 and inflammatory cytokines in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages. (*$p<0.05$, **$p<0.01$, n=3-6 per group)


A
WT
AKO
Sirt3
ATP5α
Sirt3 kd : - - - + + + - - - + + +
Sirt3 normalized to ATP-5α
WT-ctl
WT-sirt3 kd
AKO-ctl
AKO-sirt3 kd
B
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AKO
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β-Actin
Sirt3 kd : - - - + + + - - - + + +
LPS: + + + + + + + + + + + +
Cox2 normalized to β-Actin
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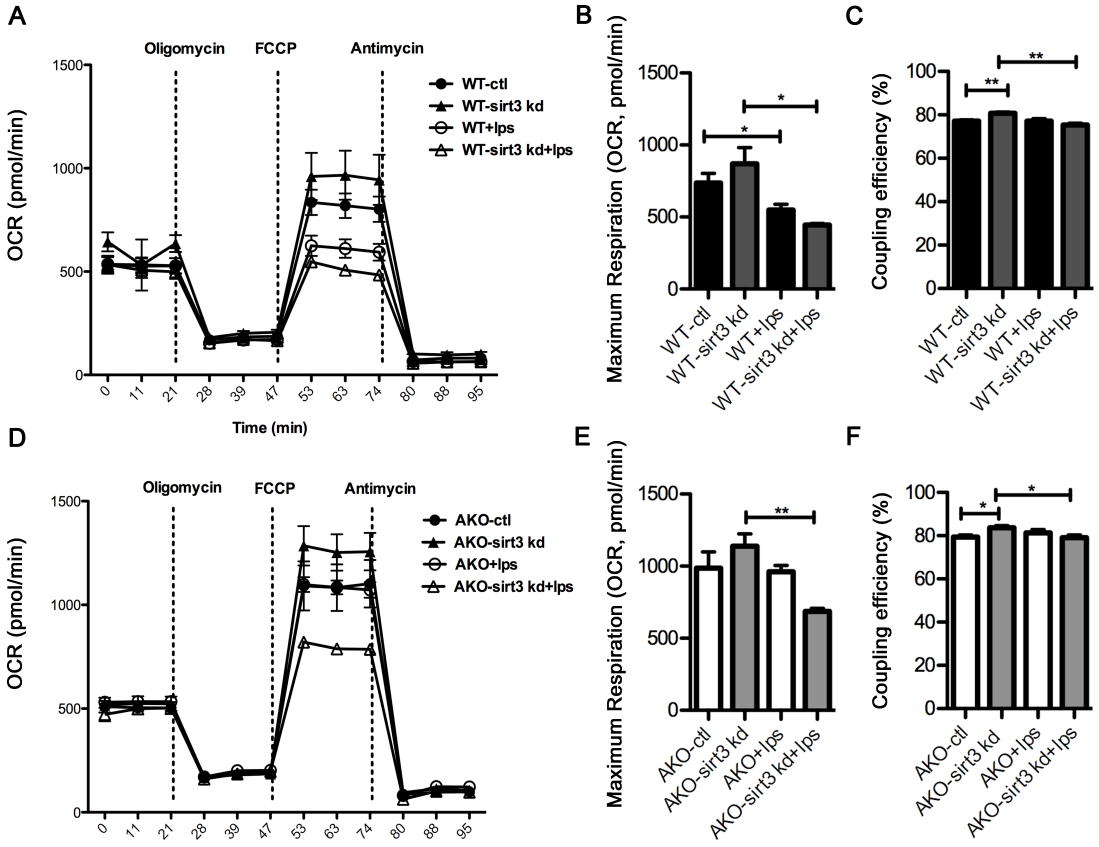
AKO
iNOS
β-Actin
Sirt3 kd : - - - - - - + + +
LPS+IFNγ: + + + + + + + + +
iNOS normalized to β-Actin
D
Relative mRNA expression
Sirt3
Mcp1
Tnfα
Mmp9
WT-ctl
WT-sirt3
AKO-ctl
AKO-sirt3

SIRT3 expression mediates the protective effect of FABP4/aP2 deficiency on LPS induced mitochondrial dysfunction. Genetic ablation of FABP4/aP2 has been shown to protect macrophages from LPS dependent loss of maximum respiration capacity, independent of uncoupling protein 2 expression (22). In order to determine if the increased expression of SIRT3 in FABP4/aP2$^{-/-}$ macrophages could mediate the suppression of LPS induced mitochondrial dysfunction, both WT-sirt3kd and AKO-sirt3kd, as well as their control cells, were treated with LPS for 6 hours and cellular respiration was measured. Consistent with our previous report (22), LPS treatment induced a decrease of maximum respiration in wild type cells, while FABP4/aP2$^{-/-}$ macrophages were protected from this effect (Figures 4A-B and D-E). Knockdown of Sirt3 rendered wild type macrophages more susceptible to a LPS induced decrease of maximum respiration (Figure 4B). Moreover, knockdown of Sirt3 in FABP4/aP2$^{-/-}$ macrophages led to a similar reduction of maximum respiration by LPS treatment, thus eliminating the protection seen in the FABP4/aP2$^{-/-}$ macrophages (Figure 4E). Interestingly, coupling efficiency was increased after Sirt3 knockdown in wild type and FABP4/aP2$^{-/-}$ macrophages and decreased in Sirt3 knockdown cells following LPS treatment (Figures 4C and F). No difference of basal respiration or ATP turnover was observed (data not shown). Therefore, up regulation of SIRT3 in FABP4/aP2$^{-/-}$ macrophages is responsible for changes in mitochondrial respiration in response to LPS treatment.

Figure 4 - SIRT3 expression protects macrophages from LPS induced mitochondrial dysfunction.

(A) Oxygen consumption rate in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM). (B) Maximum respiration in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (C) Coupling efficiency in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (D) Oxygen consumption rate in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM). (E) Maximum respiration in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (F) Coupling efficiency in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (*$p<0.05$, **$p<0.01$, n=5 per group)

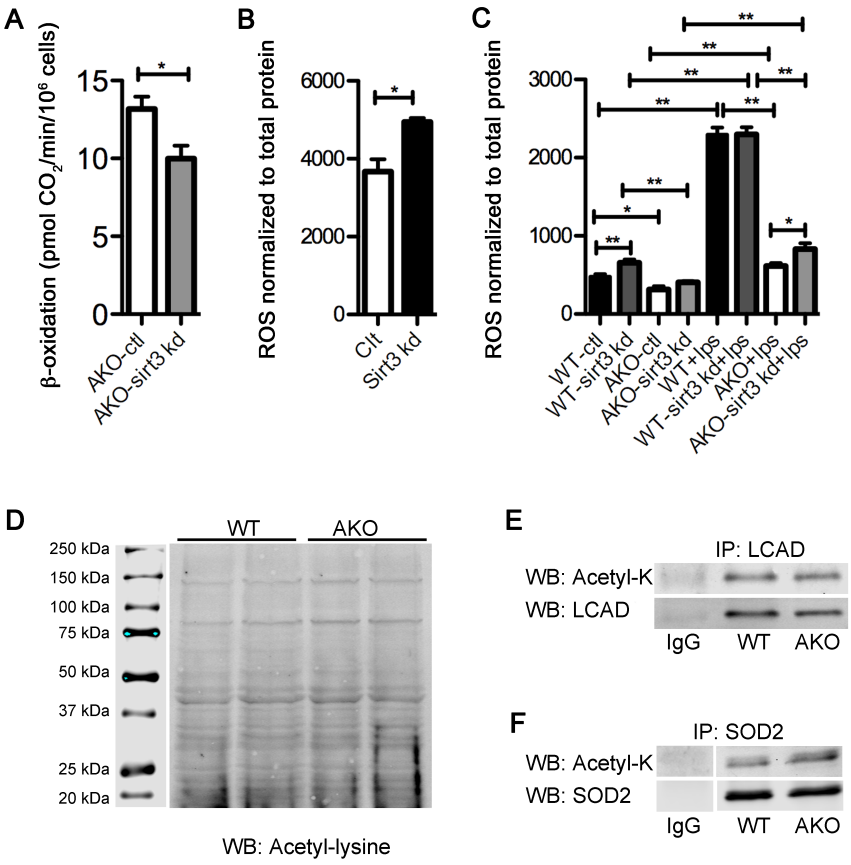

A
Oligomycin
FCCP
Antimycin
WT-ctl
WT-sirt3 kd
WT+lps
WT-sirt3 kd+lps
OCR (pmol/min)
Time (min)
B
Maximum Respiration (OCR, pmol/min)
WT-ctl
WT-sirt3 kd
WT+lps
WT-sirt3 kd+lps
C
Coupling efficiency (%)
D
AKO-ctl
AKO-sirt3 kd
AKO+lps
AKO-sirt3 kd+lps
E
F

SIRT3 expression is responsible for fatty acid oxidation and ROS production in macrophages independent of lysine acetylation changes. Among the seven members in the sirtuin family, SIRT3 plays a major role in regulating mitochondrial function by affecting various aspects of mitochondrial metabolism, including mitochondrial respiration, fatty acid oxidation, and the tricarboxylic acid cycle flux (TCA cycle) (33-35). Consistent with the report that SIRT3 regulates fatty acid oxidation (34), Sirt3 knockdown in FABP4/aP2$^{-/-}$ macrophages decreased fatty acid oxidation (Figure 5A). Another prominent role of SIRT3 is controlling reactive oxygen species (ROS) production (33). Consistently, knockdown of Sirt3 increased ROS production in Raw264.7 and wild type macrophages (Figure 5B and C). Since our previous work has shown that FABP4/aP2$^{-/-}$ macrophages have a significantly lower level of oxidative stress (22), we speculate that the increased SIRT3 expression may be responsible for the decreased ROS level. Consistent with our previous report, the ROS level was significantly lower in FABP4/aP2$^{-/-}$ macrophages compared to that in the wild type (Figure 5C). Upon LPS stimulation, there was a significant increase of ROS in wild type, but not FABP4/aP2$^{-/-}$ macrophages. Importantly, upon LPS stimulation, AKO-sirt3kd cells had a significant increase in ROS production compared to AKO macrophages, however the ROS levels were still considerably lower than that in wild type macrophages in response to LPS stimulation (Figure 5C). These data indicate up regulation of SIRT3 is at least partially responsible for the decreased ROS in FABP4/aP2$^{-/-}$ macrophages in

response to LPS treatment. Despite the increased SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages, we did not detect any substantive difference in the mitochondrial acetylome or the acetylation of classic SIRT3 targets, LCAD and SOD2 (Figures 5D-F).

Figure 5 - Increased SIRT3 expression mediates increased β-oxidation and decreased oxidative stress in FABP4/aP2$^{-/-}$ macrophages independent of protein acetylation.

(A) β-oxidation measured in AKO-ctl and AKO-sirt3 kd macrophages. (B) ROS measured by H_2DCFDA in Control and Sirt3 kd Raw264.7 macrophages. (C) ROS measured by H_2DCFDA in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (D) Mitochondrial protein acetylation in WT and AKO macrophages. (E) Analysis of acetylated and total LCAD following immunoprecipitation from WT and AKO macrophages. (F) Evaluation of acetylated and total SOD2 immunoprecipitated from WT and AKO macrophages. (*$p<0.05$, **$p<0.01$, n=3-6 per group)


A
β-oxidation (pmol CO2/min/10^6 cells)
15
10
5
0
*
AKO-ctl
AKO-sirt3 kd
B
ROS normalized to total protein
6000
4000
2000
0
*
Clt
Sirt3 kd
C
ROS normalized to total protein
3000
2000
1000
0
WT-ctl
WT-sirt3 kd
AKO-ctl
AKO-sirt3 kd
WT+lps
WT-sirt3 kd+lps
AKO+lps
AKO-sirt3 kd+lps
D
WT
AKO
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
WB: Acetyl-lysine
E
IP: LCAD
WB: Acetyl-K
WB: LCAD
IgG
WT
AKO
F
IP: SOD2
WB: Acetyl-K
WB: SOD2
IgG
WT
AKO

Discussion

Insulin resistance in peripheral tissues such as liver, adipose, and muscle is a major feature in the pathogenesis of cardiovascular disease, fatty liver, and type 2 diabetes (1,5). The progression of insulin resistance is accompanied with macrophage infiltration and chronic inflammatory activation in multiple tissues (12,36-38). Mouse models which either reduce macrophage recruitment in adipose tissue or suppress macrophage inflammation have been show to protect the animal from high fat diet induced insulin resistance (39-41). One of the most well characterized mouse models that affect these inflammatory events is genetic deletion of FABP4/aP2. Despite a similar level of adiposity, FABP4/aP2$^{-/-}$ mice are protected from diet induced insulin resistance (42). Interestingly, macrophage specific deletion of FABP4/aP2 is sufficient to prevent the development of atherosclerosis in the ApoE-deficient mouse model (14). More detailed studies have shown that loss of FABP4/aP2 could suppress inflammatory signaling in macrophages, which is a major reason for the metabolic benefits of FABP4/aP2 deficiency (13,16,43,44). Recent work from our laboratory has shown that induction of UCP2 expression is a major mediator for the decreased inflammation and endoplasmic reticulum stress responses observed in FABP4/aP2$^{-/-}$ macrophages (22). However, up-regulation of UCP2 does not explain the increased fatty acid oxidation and improved mitochondrial function in FABP4/aP2$^{-/-}$ macrophages. Previous reports demonstrating a MUFA-SIRT1-PGC1α-SIRT3 axis, as well as our own observation that monounsaturated fatty acids are specifically increased in FABP4/aP2$^{-/-}$ macrophages, led us to

speculate that SIRT3 expression might be altered in FABP4/aP2$^{-/-}$ macrophages (26,27). Indeed, the results herein showed a dramatic increase of SIRT3 protein, but not mRNA, following loss of macrophage FABP4/aP2. Additionally, silencing of Sirt3 in FABP4/aP2$^{-/-}$ macrophages decreased fatty acid oxidation. More importantly, our results also demonstrated for the first time that MUFAs, but not palmitate, could induce SIRT3 expression in macrophages. By knocking down Sirt3 in macrophages, the results here revealed an important role of SIRT3 in the suppression of macrophage inflammation. Induction of SIRT3 is required for the anti-inflammatory role of palmitoleate, as knockdown of Sirt3 compromised the reduction of Cox2 expression caused by palmitoleate treatment.

In parallel with the identification of UCP2 as a major mediator of the decreased inflammation in FABP4/aP2$^{-/-}$ macrophages, the loss of SIRT3 also restored the inflammation in FABP4/aP2 deficient cells similar to wild type macrophages. Interestingly, SIRT3 expression has been shown to be correlated with UCP2 levels in hepatocytes cultured in high glucose and in this system, inhibition of SIRT3 also leads to decreased UCP2 expression (45). Therefore, it is likely that SIRT3 acts as an upstream regulator of UCP2, and exerts some of its action by suppressing inflammation in macrophages through UCP2.

SIRT3 localizes primarily to mitochondria and regulates the activity of a number of enzymes involved in some major metabolic pathways, including tricarboxylic acid cycle, the urea cycle, and fatty acid metabolism (33-35). Given the

prominent role of SIRT3 in mitochondrial function, it was not surprising to find that the up-regulation of SIRT3 is an important contributor to the improved mitochondrial function observed in FABP4/aP2$^{-/-}$ macrophages (22). While FABP4/aP2$^{-/-}$ macrophages are protected from LPS induced loss of maximum respiration capacity, knockdown of SIRT3 potentiated LPS induced mitochondrial dysfunction. Consistent with SIRT3's role as a suppressor of oxidative stress, loss of SIRT3 increased ROS production both basally and in response to LPS in macrophages.

Paradoxically, despite the dramatic up-regulation of SIRT3, we were not able to detect any difference of global mitochondrial protein lysine acetylation or in the acetylation status of classic targets of SIRT3 such as LCAD and SOD2. One potential explanation is that SIRT3 exerts its role in inflammation, ROS control, and mitochondrial function through a mechanism other than protein deacetylase activity in macrophages (46). It is also possible that the acetylation status of the SIRT3 targets analyzed here is affected by other pathways in addition to SIRT3. Generally, SIRT3 is considered as a ROS suppressor mainly by regulating SOD2 acetylation status (47). However, we did not detect any difference of SOD2 expression or acetylation between wild type and FABP4/aP2$^{-/-}$ macrophages (Figure 5). While we speculate that SIRT3 serves as an oxidative stress suppressor in macrophages by regulating UCP2 expression, further work is required to validate this point.

In summary, the results presented herein demonstrate that SIRT3 is up-regulated in FABP4/aP2 deficient macrophages, potentially via increased levels of palmitoleic acid. The increased SIRT3 expression mediates decreased inflammation and intracellular ROS as well as the improved mitochondrial function in FABP4/aP2$^{-/-}$ macrophages. However, increased SIRT3 expression did not affect global acetylation status of mitochondrial protein in FABP4/aP2$^{-/-}$ macrophages.

Acknowledgement

The authors would like to thank the members of the Bernlohr laboratory for helpful discussions during the study and preparation of the manuscript. We would also like to thank Drs. Douglas Mashek and Salmanan Kahn for several helpful suggestions. The support of the Minnesota Supercomputing Institute is gratefully acknowledged.

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CHAPTER FOUR

Regulation of the Adipose PPARγ-FABP4-UCP2 Axis After Bariatric Surgery

Cyrus Jahansouz*, Hongliang Xu*, Ann V. Hertzel, Rocio Avilo Foncea, Federico J. Serrot, Nicholas Kvalheim, Girish Luthra, Kristin Ewing RD, Daniel B. Leslie Sayeed Ikramuddin and David A. Bernlohr

This chapter contains an yet unpublished original research article.

Hongliang Xu performed experiments in figure1 (Panel e, f, and g), figure 2, figure3d, and figure S1 (Panel g, and h).

Summary

Bariatric surgery remains the most effective treatment for weight loss and type 2 diabetes mellitus, however the mechanism(s) responsible has remained elusive. Herein we identify a novel mechanism that Vertical Sleeve Gastrectomy (VSG) and Roux-en-Y Gastric Bypass (RYGB) acutely downregulate adipose tissue expression of *PPARγ* and its downstream targets, including Fatty Acid Binding Protein 4 (*FABP4*), and upregulate adipose Uncoupling Protein 2 (*UCP2*) expression. In contrast, matched hypocaloric restriction markedly increased adipose *PPARγ* expression, with no change in adipose *FABP4* and *UCP2* expression. These post-surgical alterations are mechanistically important since reducing adipose *PPARγ* improves insulin sensitivity, as do the traditional PPARγ activating thiazolidinediones. Furthermore, decreased *FABP4* has been demonstrated to improve insulin sensitivity, while increased *UCP2* levels function to reduce reactive oxygen species, thus promoting an anti-inflammatory phenotype. Hemoglobin A1c (HbA1c) levels predicted expression of *PPARγ*, *FABP4* and *UCP2*. Higher preoperative hemoglobin A1c levels correlated with a diminished capacity to reduce adipose *PPARγ* and FABP4 expression, and increase UCP2 expression. Altogether, our data suggest that bariatric surgery acutely reduces adipose tissue *PPAR*g and *FABP4* expression, while increasing *UCP2* levels, leading to the early resolution of insulin resistance. Importantly, these changes occur prior to substantial weight loss and are independent of

caloric restriction, thus ultimately driving the surgical-dependent metabolic improvements.

Introduction

Globally, the prevalence of obesity has been on the rise with significant impact on health care (1). The metabolic pathologies associated with obesity include cardiovascular disease, non-alcoholic fatty liver disease and type 2 diabetes mellitus (T2DM) (2, 3). Adipose tissue, as an endocrine organ, plays an important role in the pathogenesis of obesity-related diseases. The accumulation of proinflammatory immune cells in adipose tissue during the development of obesity leads to increased secretion of inflammatory cytokines such as TNFα, MCP1, and IFNγ (4, 5). Hyperplastic adipocytes secrete increased levels of fatty acids and adipokines such as fatty acid binding protein 4 (FABP4; also known as aP2), resistin, LCN2, and leptin (6). These factors from adipocytes and immune cells synergistically lead to insulin resistance both locally and globally, which is key to the pathogenesis of type 2 diabetes (7).

Targeted methods to reduce insulin resistance have been a mainstay for the medical management of T2DM. To this end, the thiazolidinedione (TZD) class of anti-diabetic medications is widely used. TZDs serve as activating ligands of Peroxisome Proliferator-Activated Receptor γ (PPARγ), a nuclear hormone receptor involved in adipocyte and macrophage metabolism (8). PPARγ is most abundantly expressed in adipose tissue, with both isoforms present, similar to rodents. PPARγ expression is elevated in obese individuals with an increased ratio of PPAR γ2/γ1 (8). PPARγ activation leads to increased insulin sensitivity albeit at the cost of additional weight gain (9). Conversely, decreasing PPARγ

levels also increases insulin sensitivity. For example, Miles *et al*. have shown in mice that heterozygous deficiency of PPARγ improves insulin sensitivity indicating the mechanistic complexity linking PPARγ activity to insulin sensitivity (10). Interestingly, a downstream target for PPARγ, FABP4, has been studied as an intracellular protein with a proinflammatory nature, but recently has been identified as a secretory protein (11, 12). Multiple human and mouse genetic studies have implicated FABP4 in the chronic inflammation of adipose tissue and impaired systemic insulin sensitivity and glucose homeostasis (13, 14). Furthermore, we have recently shown that the suppression of inflammation resulting from the attenuation of FABP4 expression relies on the induction of mitochondrial uncoupling protein 2 (UCP2), a protein previously identified as a major suppressor of reactive oxygen species (15).

Despite all the medications available, the most effective treatment to achieve acute and sustained weight loss along with improved insulin sensitivity for obese patients is bariatric surgery such as vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB) (16, 17). Indeed, by one-week post surgery when minimal weight has been lost, insulin sensitivity is significantly increased (18). However, the mechanism(s) responsible for such rapid metabolic changes after surgery has been poorly characterized and remain largely unknown. Studies have indicated a more complicated modulation beyond simple restriction is responsible for the dramatic efficacy of the surgery. For instance, gut microbiota

shifts, changes of bile acids, short–chain fatty acids, and gut hormones such as glucagon-like peptide 1 (GLP1) and Ghrelin have all been implicated as mechanistic explanations for the effectiveness of the surgery (19–25). However, detailed study of these proposed mechanisms remain rather unsatisfying.

Herein, we propose a novel mechanism post-bariatric surgery affecting adipose tissue PPARγ-FABP4-UCP2 axis as an important target for regulating obesity-dependent metabolic disease. Adipose PPARγ expression and activity as well as FABP4 expression are dramatically reduced with a concomitant increase in *UCP2* as early as one week following bariatric surgery. These changes occur independent of hypocaloric restriction and precede substantial weight loss.

RESULTS

Expression of PPARγ and downstream targets decrease after bariatric surgery. VSG was performed on fifteen female and five male patients (Supplemental Table 1). Patients tended to be prediabetic with a narrow degree of insulin resistance as interpreted by HOMA-IR. Only two diabetic patients required supplemental medication for glucose control (Metformin and Linagliptin), thus limiting the confounding of analysis and interpretation of subsequent data. One week post-VSG, weight loss ranged from 0.8 kg to 6.8 kg (Fig. 1a). As expected, patients who were prediabetic or diabetic had higher initial HOMA-IR levels (5.28 +/- 0.87) and observed a steeper decline in these levels post surgery (3.39 +/- 0.54; $P = 0.02$). Only one patient experienced an increase in HOMA-IR after surgery (Fig. 1b).

To explore the early changes in adipose tissue metabolism post surgery, subcutaneous adipose tissue (SAT) biopsies prior to and one week following the VSG were analyzed. A significant reduction in adipose tissue *PPARγ* mRNA levels was determined, decreasing on average by 60% (Fig. 1c). Interestingly, the one patient who observed an increase in HOMA-IR post-surgery also had an increase in adipose tissue *PPARγ* mRNA levels (Fig. 1b,c). Next, expression of several well-established PPARγ targets, including *FABP4*, *PDE3b*, *ATGL*, *CD36*, and *SCD* were evaluated. Consistent with the decrease in *PPARγ* mRNA levels, expression of all targets evaluated decreased significantly, except for *SCD*,

which exhibited a trend towards a decreased level (Fig. 1d). Assessment of PPARγ protein levels indicated a similar trend (*P*=0.09), with each patient on average decreasing by nearly 60% (Fig. 1e). Furthermore, protein levels of CD36 decreased significantly, whereas another PPARγ target, Perilipin 1, trended downwards (Fig. 1e).

We next sought to identify which cell population(s) is responsible for the decreases in expression of PPARγ and gene targets of PPARγ. Thus we performed collagenase digestion of adipose tissue biopsies to obtain a primary adipocyte fraction (ADIP) and a stromal vascular fraction (SVF). Interestingly, mRNA levels of *PPARγ* and its downstream targets were significantly decreased or trending towards decreased levels in both fractions. One notable exception was *PDE3B* mRNA levels in SVF, which nearly tripled in contrast to that in ADIP in which mRNA levels decreased by half (Fig. 1f,g). This may indicate a key difference in the response of immune cells and adipocytes following bariatric surgery.

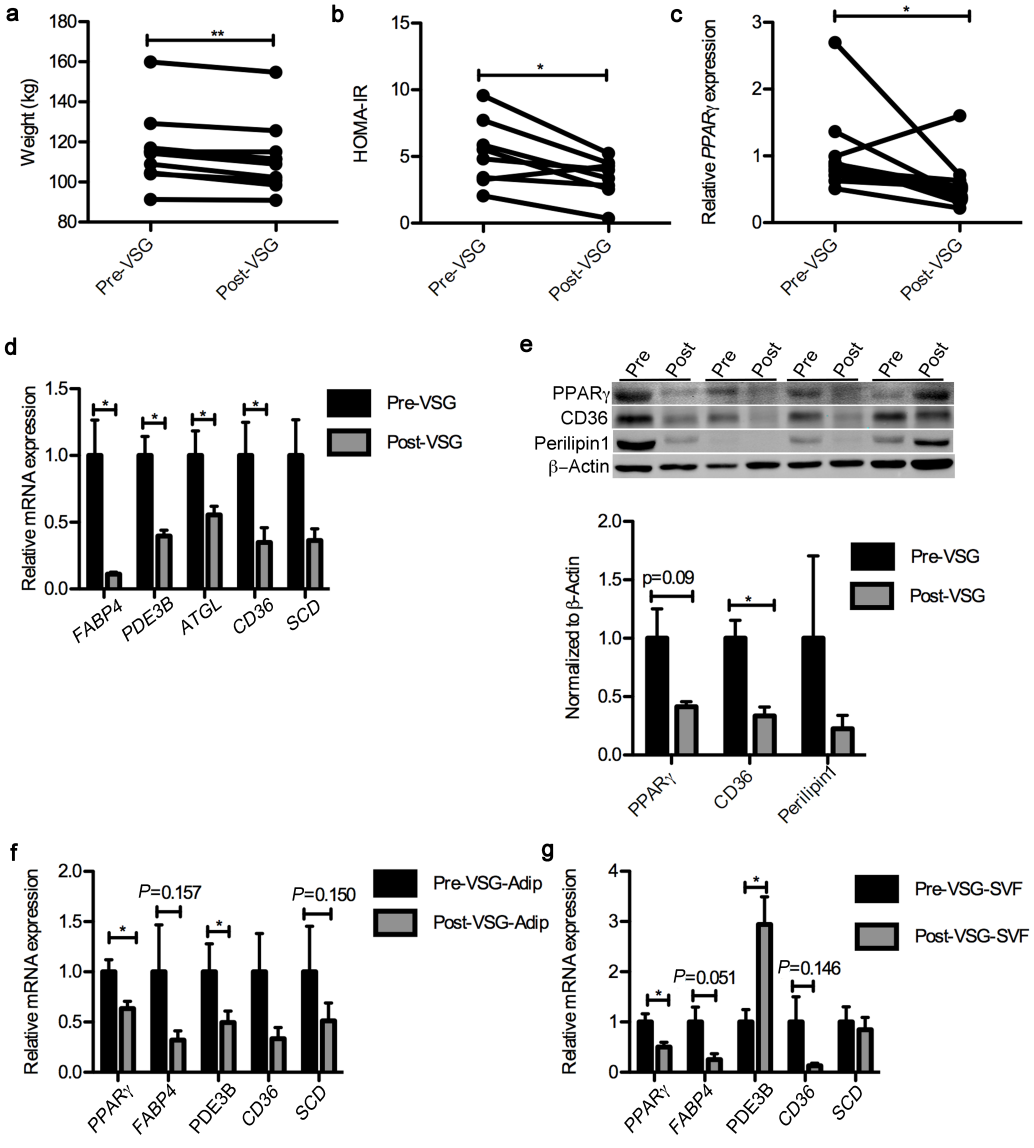
	VSG Preop	VSG Postop
N	20	-
Gender (M/F)	5/15	-
Age (years)	40.0 +/- 2.2	-
Weight (kg)	122.2 +/- 7.6	*118.2 +/- 7.7
Weight Lost (%)	-	3.5 +/- 0.5
HbA1c (%)	5.9 +/- 0.2	-
Prediabetes or T2DM	8	-
Glucose (mg/dL)	103.6 +/- 7.2	88.1 +/- 3.3
Insulin (mU/L)	12.3 +/- 1.9	11.5 +/- 1.4
HOMA-IR	3.3 +/- 0.6	*2.9 +/- 0.4
Free Fatty Acids (mmol/L)	1.06 +/- 0.07	*0.81+/- 0.09

Supplementary Table 1 – Baseline and postoperative characteristics of patients who underwent VSG.

* denotes significantly different from pre surgery, $P < 0.05$. Values are means +/- SEM. Abbreviations: Preop = preoperative value; Postop = postoperative value

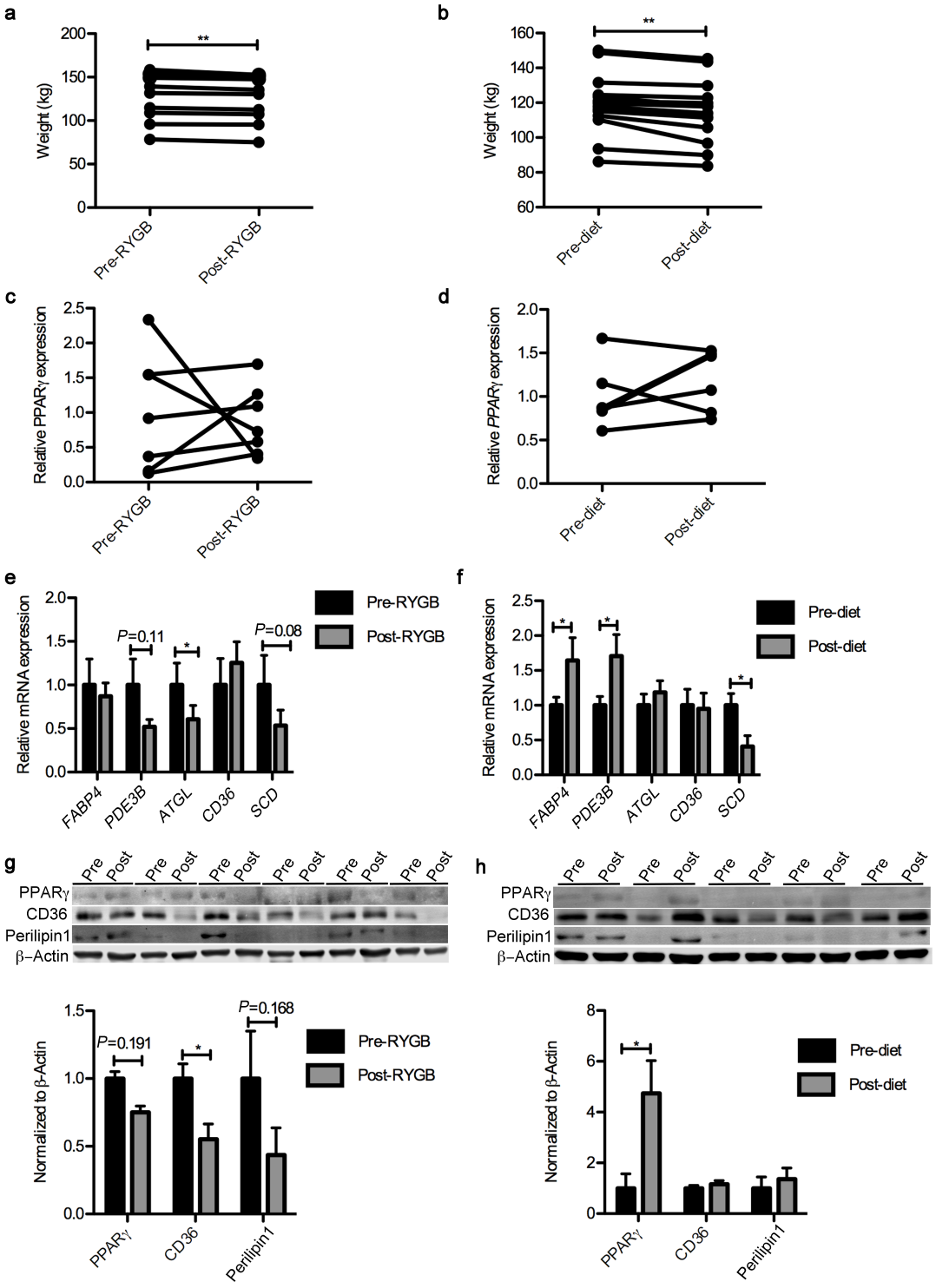
Figure 1- VSG down regulates PPARγ and PPARγ target expression in adipose tissue.

(a) Body weight of patients before (pre) and one week after (post) VSG (*n*=20). **(b)** HOMA-IR of patients before and one week after VSG. **(c)** Relative *PPARγ* mRNA levels in subcutaneous adipose tissue before and one week after VSG (*n*=12). **(d)** Relative mRNA levels of *FABP4* (fatty acid binding protein4), *PDE3B* (phosphodiesterase 3B), *ATGL* (adipose triglyceride lipase), *CD36* (cluster of differentiation 36), *SCD* (stearoyl-CoA desaturase 1) in subcutaneous adipose tissue before and one week after VSG. **(e)** Protein level of PPARγ, CD36, and Perilipin 1 measured by western blot in adipose tissue of patients before and one week after VSG (*n*=12). **(f)** mRNA levels of *PPARγ, FABP4, PDE3B, CD36,* and *SCD* in primary adipocyte fraction of subcutaneous adipose tissue of patients before and one week after VSG (*n*=8). **(g)** mRNA levels of *PPARγ, FABP4, PDE3B, CD36,* and *SCD1* in stromal vascular fraction of subcutaneous adipose tissue of patients before and one week after VSG (*n*=8).


a
Weight (kg)
180
160
140
120
100
80
**
Pre-VSG
Post-VSG
b
HOMA-IR
15
10
5
0
*
Pre-VSG
Post-VSG
c
Relative PPARγ expression
3
2
1
0
*
Pre-VSG
Post-VSG
d
Relative mRNA expression
1.5
1.0
0.5
0.0
*
FABP4
PDE3B
ATGL
CD36
SCD
Pre-VSG
Post-VSG
e
Pre
Post
PPARγ
CD36
Perilipin1
β–Actin
Normalized to β-Actin
2.0
1.5
1.0
0.5
0.0
p=0.09
*
PPARγ
CD36
Perilipin1
Pre-VSG
Post-VSG
f
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
P=0.157
*
P=0.150
PPARγ
FABP4
PDE3B
CD36
SCD
Pre-VSG-Adip
Post-VSG-Adip
g
Relative mRNA expression
4
3
2
1
0
*
P=0.051
*
P=0.146
PPARγ
FABP4
PDE3B
CD36
SCD
Pre-VSG-SVF
Post-VSG-SVF

Supplementary Figure 1 - Expression of *PPARγ* and downstream targets in subcutaneous adipose tissue of patient undergone RYGB or caloric restriction.

(a) Body weight of patients before (pre) and one week after (post) RYGB (*n*=13). **(b)** Body weight of patients before (pre) and one week after (post) caloric restriction (*n*=14). **(c)** Relative *PPARγ* mRNA levels in subcutaneous adipose tissue before and one week after RYGB. **(d)** Relative *PPARγ* mRNA levels in subcutaneous adipose tissue before and one week after caloric restriction. **(e)** Relative mRNA levels of *FABP4, PDE3B, ATGL, CD36,* and *SCD* in subcutaneous adipose tissue before and one week after RYGB. **(f)** mRNA levels of *FABP4, PDE3B, ATGL, CD36,* and *SCD* in the subcutaneous adipose tissue of patients before and one week after caloric restriction. **(g)** Protein levels of PPARγ, CD36, and Perilipin 1 measured by western blot in adipose tissue of patients before and one week after RYGB. **(h)** Protein levels of PPARγ, CD36, and Perilipin 1 measured by western blot in adipose tissue of patients before and one week after caloric restriction.


a
Weight (kg)
200
150
100
50
0
**
Pre-RYGB
Post-RYGB
b
Weight (kg)
160
140
120
100
80
60
**
Pre-diet
Post-diet
c
Relative PPARγ expression
2.5
2.0
1.5
1.0
0.5
0.0
Pre-RYGB
Post-RYGB
d
Relative PPARγ expression
2.0
1.5
1.0
0.5
0.0
Pre-diet
Post-diet
e
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
P=0.11
*
P=0.08
Pre-RYGB
Post-RYGB
FABP4
PDE3B
ATGL
CD36
SCD
f
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Pre-diet
Post-diet
FABP4
PDE3B
ATGL
CD36
SCD
g
Pre
Post
PPARγ
CD36
Perilipin1
β–Actin
Normalized to β-Actin
1.5
1.0
0.5
0.0
P=0.191
*
P=0.168
Pre-RYGB
Post-RYGB
PPARγ
CD36
Perilipin1
h
Pre
Post
PPARγ
CD36
Perilipin1
β–Actin
Normalized to β-Actin
8
6
4
2
0
*
Pre-diet
Post-diet
PPARγ
CD36
Perilipin1

Surgery acts independently from caloric restriction in adipose tissue

Multiple studies have indicated normalization of plasma glucose and insulin levels as early as one week after bariatric surgery, prior to significant weight loss (26). Also, it is clinically accepted that bariatric surgery acts in a manner independently and beyond that from hypocaloric restriction (HC Diet) (16, 17, 27). In order to assess whether the observed post-surgical effects on PPARγ were independent of the caloric restriction following the surgery, we performed a series of analyses on a subset of our previous controlled trial of patients who were randomized to RYGB or hypocaloric restriction. Demographics for these two cohorts have been previously published in Jahansouz *et al* (18).

As shown in Supplementary Fig. 1a and 1b, weight loss per patient after each intervention was similar, with no consistent change in subcutaneous adipose *PPARγ* mRNA levels after either intervention (Supplementary Fig. 1c,d). More detailed analysis of gene expression of downstream PPARg targets revealed reductions in *PDE3B* and *ATGL* after RYGB, but increases in *PDE3B* and *FABP4* in HC Diet. *SCD* decreased after both interventions, but to a greater extent after caloric restriction (Supplementary Fig. 1e,f). Protein levels of PPARγ and its downstream targets, CD36 and Perilipin, decreased or trended downward after RYGB, similar to that seen post-VSG (Supplementary Fig. 1g). In contrast, PPARγ protein levels increased five-fold after hypocaloric restriction, without changes to CD36 and Perilipin 1 (Supplementary Fig. 1h). In summary, the

above data indicate RYGB and VSG have similar effects on expression of PPARγ and its targets, which is independent of caloric restriction.

FABP4 levels decrease after bariatric surgery, but not after hypocaloric restriction

To further assess the effects of bariatric surgery on FABP4 expression, we measured the adipose intracellular FABP4 protein level. As shown in Fig. 2a, FABP4 decreased on average by nearly 40% per patient at one-week post-surgery. While *FABP4* mRNA levels did not significantly change in the RYGB population, protein levels dropped by nearly 20% (Fig. 2b). In contrast, FABP4 protein levels did not change in the HC diet patients (Fig. 2c). FABP4 has also been identified as an adipokine that could contribute to systemic insulin resistance through actions on liver and pancreatic b cells (28, 29). Therefore, we also measured the serum levels of FABP4 in the three cohorts one-week after intervention. While serum levels of FABP4 did not change in either the VSG or RYGB cohorts, they were increased about 30% in the HC diet cohort (Fig. 2d). Although randomization was used to divide the RYGB and hypocaloric diet cohorts, it is unclear why serum FABP4 levels started lower in the diet cohort. It is worth noting that the RYGB cohort was a more diabetic group than the VSG cohort with higher HbA1c, HOMA-IR, and diabetic medication requirements. Thus, it was not surprising to observe the higher starting levels of FABP4 in this population relative to the VSG patients. Upon further analysis, a distinction was

apparent in this population of predominantly diabetic patients in the RYGB group; patients who started with FABP levels greater than 100 ng/mL all experienced a decrease in circulating levels one week after surgery (175.9 +/- 14.3 ng/mL to 106.4 +/- 10.3 ng/mL; *P*=0.003). No discernible change was observed in patients with pre-surgical circulating FABP4 levels less than 100 ng/mL.

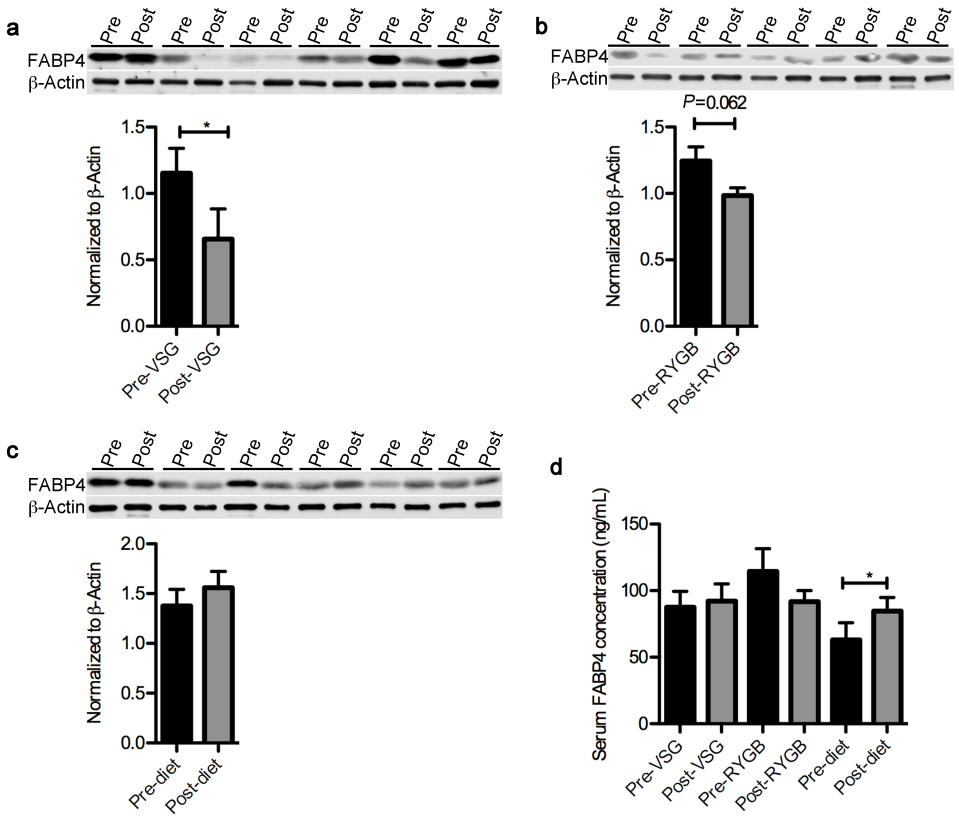


Figure 2 - Expression of FABP4 decreases in subcutaneous adipose tissue of patients following bariatric surgery, independent of caloric restriction.

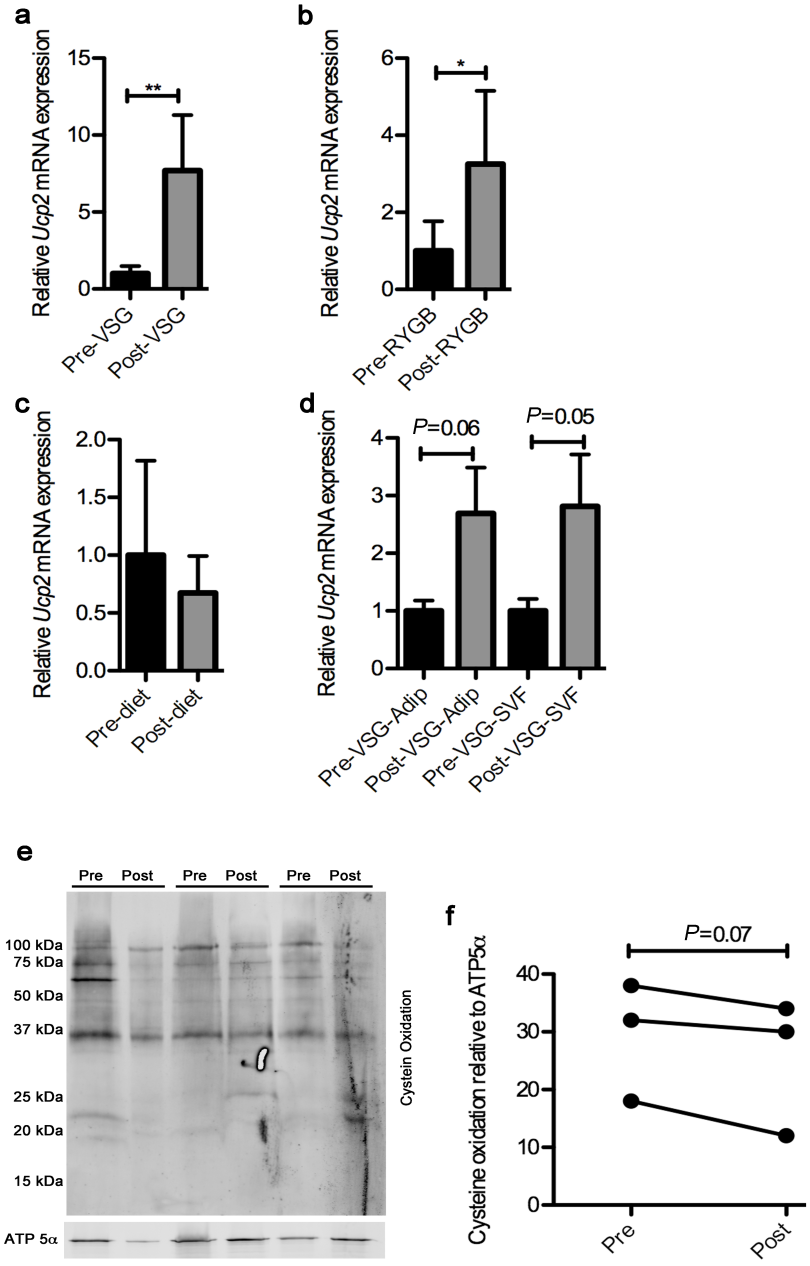
(a) FABP4 protein levels in subcutaneous adipose tissue of patients before and after VSG determined by western blot (*n*=12). **(b)** FABP4 protein levels in subcutaneous adipose tissue of patients before and one week after RYGB determined by western blot (*n*=13). **(c)** FABP4 protein levels in subcutaneous adipose tissue of patients before and one week after caloric restriction diet determined by western blot (*n*=14). **(d)** Serum FABP4 level measured by ELISA (VSG: *n*=12; RYGB: *n*=13; HC Diet: *n*=14).

UCP2 levels increase and cysteine oxidation decreases in adipose tissue after bariatric surgery

We previously identified an inverse relationship between FABP4 and UCP2 expression. Importantly, we demonstrated that the improved insulin sensitivity acquired from decreased FABP4 expression is dependent on the upregulation of UCP2. Therefore, *UCP2* mRNA was assessed in subcutaneous adipose tissue biopsies of VSG patients before and one week after surgery. As shown in Fig. 3a, *UCP2* expression increased approximately seven fold following VSG. Consistent with this observation, *UCP2* expression was also significantly increased in adipose tissue one week post RYGB, but not in adipose from the HC diet cohort (Fig. 3b,c). In order to identify which cell population(s) was responsible for the increased *UCP2* expression, we measured the mRNA levels of *UCP2* in both primary adipocytes and the stromal vascular fraction from SAT. Consistent with the observed decreased levels of FABP4, *UCP2* expression was increased in both fractions (Fig. 3d). UCP2 has consistently been shown to function via a reduction of reactive oxygen species and we have also demonstrated reduced ROS in FABP4 knockout macrophages (15, 30). Furthermore, since ROS leads to oxidation of cysteine residues, we also have identified reduced cysteine oxidation in FABP4 knockout macrophages (Steen *et al*. unpublished). Therefore, we also quantified cysteine oxidation in SAT of patients undergoing the VSG. Cysteine oxidation was consistently decreased one week after surgery (Fig. 3e-f).

Figure 3 - Expression of *UCP2* increases in subcutaneous adipose tissue of patients following bariatric surgery, independent of caloric restriction.

(a-c) Expression of *UCP2* mRNA in subcutaneous adipose tissue of patients before and after surgery or caloric restriction determined by qRT-PCR. (**a**) VSG (*n*=12), (**b**) RYGB (*n*=13), and (**c**) caloric restriction (*n*=14). (**d**) mRNA level of *UCP2* in the primary adipocyte fraction (Adip) and stromal vascular fraction (SVF) of subcutaneous adipose tissue of patients before and one week post-VSG (*n*=8). Cysteine oxidation in subcutaneous adipose tissue was decreased one week following VSG. (**e**) Cysteine oxidation levels in subcutaneous adipose tissue of patients before and one week after VSG determined by western blot (*n*=3). (**f**) Quantification of cysteine oxidation levels in subcutaneous adipose tissue of patients before and one week after VSG.


a
Relative Ucp2 mRNA expression
15
10
5
0
**
Pre-VSG
Post-VSG
b
Relative Ucp2 mRNA expression
6
4
2
0
*
Pre-RYGB
Post-RYGB
c
Relative Ucp2 mRNA expression
2.0
1.5
1.0
0.5
0.0
Pre-diet
Post-diet
d
Relative Ucp2 mRNA expression
4
3
2
1
0
P=0.06
P=0.05
Pre-VSG-Adip
Post-VSG-Adip
Pre-VSG-SVF
Post-VSG-SVF
e
Pre
Post
Pre
Post
Pre
Post
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
Cystein Oxidation
ATP 5α
f
Cysteine oxidation relative to ATP5α
40
30
20
10
0
P=0.07
Pre
Post

Preoperative HbA1c correlates with tissue gene expression changes of *PPARγ* and downstream targets

In an attempt to reconcile the difference in diabetic status between patients in our cohorts undergoing VSG and those undergoing RYGB, we pooled all surgical patient data and evaluated whether preoperative diabetic status, specifically HbA1c or HOMA-IR, may account for differences in *PPARγ* gene expression and that of its downstream targets. While HOMA-IR did not correlate with changes in *PPARγ*, HbA1c correlated well in not only predicting changes in mRNA levels of *PPARγ*, but also with several downstream targets, specifically *FABP4*, *PDE3B*, and *CD36*, while no apparent correlation was observed with either *ATGL* or *SCD* (Fig. 4a-f). Interestingly, HbA1c also correlated with *UCP2* (Fig 4g). The strongest correlation was with *PPARγ* (Fig. 4a), suggesting that patients with higher preoperative HbA1c experience smaller changes in *PPARγ* and downstream targets, at least acutely after surgery.

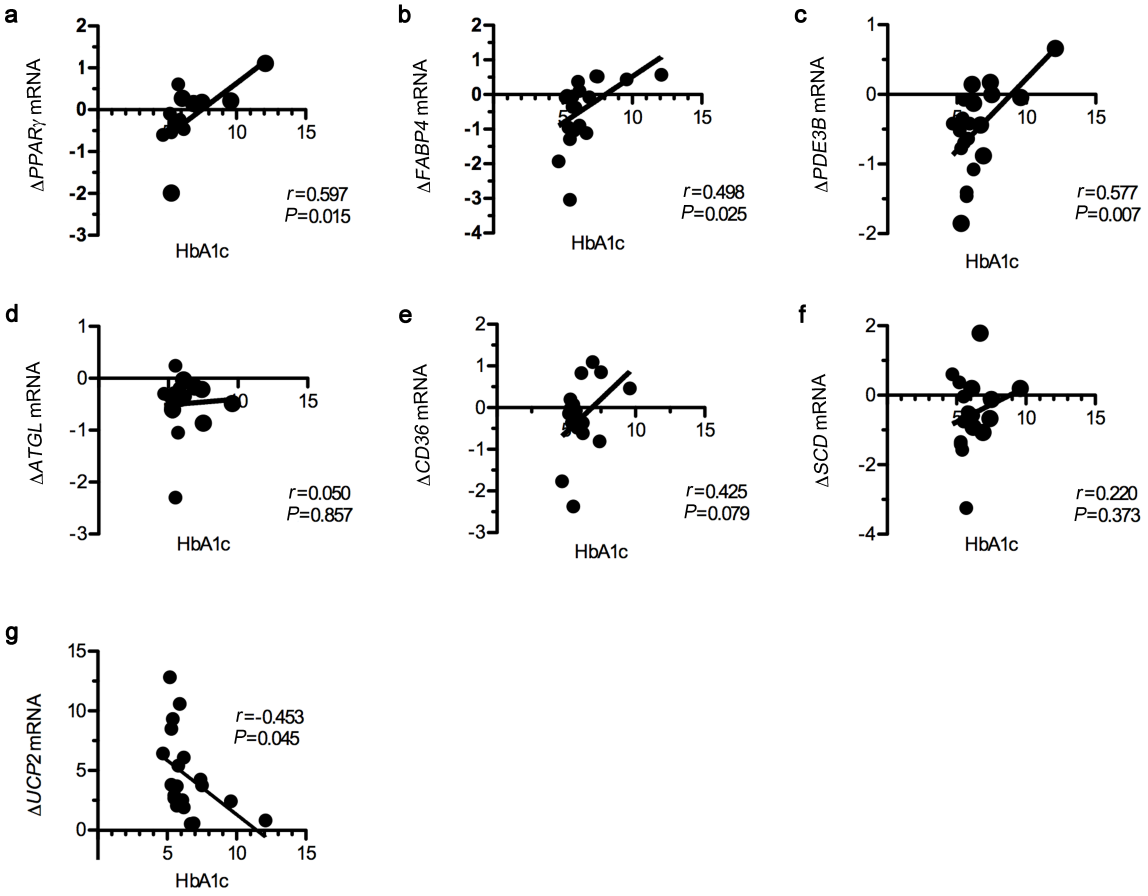


Figure 4: Preoperative HbA1c correlates with changes in adipose tissue expression of *PPARγ*, PPARγ targets and *UCP2*.

Regression analysis of preoperative HbA1c and change in mRNA expression one week after bariatric surgery. **(a)** *PPARγ*, **(b)** *FABP4*, **(c)** *PDE3B*, **(d)** *ATGL*, **(e)** *CD36*, and **(f)** *SCD*. (**g**) Regression analysis of HbA1c and change in mRNA expression of *UCP2* one week after bariatric surgery.

DISCUSSION

Obesity and T2DM will continue to have significant impacts as major sources of morbidity and economic burden(1). Despite the multitude of medications available, bariatric surgery remains the most efficacious treatment of obesity and its associated syndromes (16, 17, 26). While the mechanism(s) underlying bariatric surgery remains enigmatic, numerous proposals have been suggested, including changes in bile acids, insulin secretory capacity and islet function, gut hormones (glucagon-like peptide 1 and gastric inhibitory polypeptide), and more recently gut microbiome and vagal innervation (19–21, 24, 31). Logically, while the gastrointestinal tract is an appropriate starting point for understanding the altered physiology associated with the postsurgical state, the contribution(s) of adipose tissue biology and associated mechanistic changes remain underappreciated and poorly understood.

Notably, in this study we have identified a mechanism that may serve as a driving force behind the benefits of bariatric surgery (Fig. 5). Herein we report for the first time that following bariatric surgery, VSG or RYGB, and prior to substantial weight loss, there is a consistent decrease in expression of PPARγ as well as PPARγ downstream targets in SAT, independent of caloric restriction (Fig. 1 and Supplementary Figure 1). PPARγ is abundantly expressed in tissues and circulating cells that are directly involved with lipid metabolism (8, 32). Patients with dominant negative mutations of PPARγ are extremely insulin resistant (33).

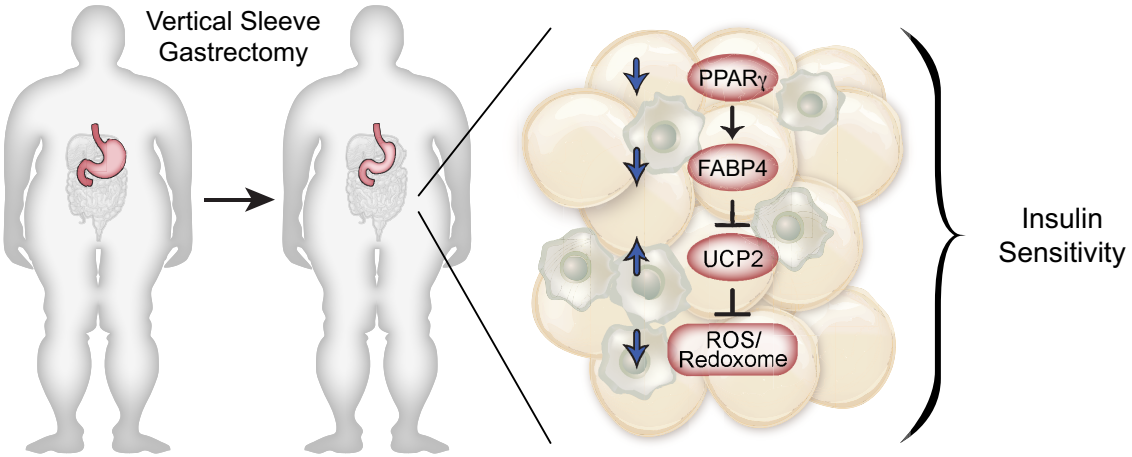


Figure 5 - Model of the acute alterations in subcutaneous adipose tissue that result from bariatric surgery. Bariatric surgery leads to acute changes (7 days) in subcutaneous adipose tissue: decreased expression of *PPARγ*, *PPARγ* targets, including *FABP4*, and increased *UCP2* expression. These changes are independent of calorie restriction and underlie the acute benefits post-surgery

In contrast, the Pro12Ala mutation in PPARγ, which decreases its transcriptional activity, improves glucose homeostasis (34). These oppositely juxtaposed reports indicate a deeper level of complexity underlying the role of PPARγ in glucose homeostasis. Following surgery, the expression of *PPARγ*, as well as two of its targets, *FABP4* and *PDE3B*, inversely correlated with HbA1c. However, compared to adipose from post-VSG, RYGB patients exhibited a smaller reduction in the expression of *PPARγ* and downstream targets. This may partially be explained by the influence of medications and/or by differing levels of insulin resistance. Thus one may speculate that chronicity of the disease state decreases the potential for reversibility of adipose tissue pathology. In support of this, reports highlight the predictive capacity of preoperative diabetic status, namely that a more severe diabetic state as well as a longer duration of diabetes portend a lower likelihood of remission after surgery (35). Preoperative BMI has not been consistently shown to predict diabetes remission (36).

A point of additional emphasis lies in the remarkable contrast in PPARγ levels in the HC Diet cohort, compared to the RYGB cohort: substantially increased protein levels of PPARγ and mRNA levels of two major PPARγ targets, *FABP4* and *PDE3B* in the HC Diet cohort. Thus, surgery may affect a distinct, PPARγ

centered mechanism to achieve metabolic benefits, different from caloric restriction. This may be of significant clinical importance since TZDs may act in direct opposition to the effects of surgery, potentially diminishing some of the associated metabolic benefits.

PPARγ regulates multiple facets of adipocyte biology and plays a critical role in mediating immune cell function. Interestingly, our results indicate that decreased *PPARγ* levels and several of its downstream targets are observed in both the adipocyte and SVF fractions. Among the targets assessed, the expression of *PDE3B* displayed a degree of discordance as mRNA levels halved in the adipocyte fraction after surgery, but increased substantially in the SVF. This distinct expression pattern of *PDE3B* following bariatric surgery supports a role for adipose tissue macrophages that has been highlighted previously by Ferranti *et al*. which favors lipolysis in adipocytes and lipid uptake by immune cells, likely macrophages, in a phagocytic role (37). Fasting has been shown to acutely induce lipid droplet formation in adipose tissue macrophages. The increased *PDE3B* in SVF may indicate increased fatty acid storage in the adipose tissue macrophages, which is consistent with previous reports (37).

Serum FABP4 levels have been identified as a hallmark for metabolic and cardiovascular disease (11, 12, 38) and have been linked to hyperinsulinemia and hepatic insulin resistance via interactions with both islet cells and

hepatocytes, respectively (28, 29). We were not able to observe a consistent decrease in circulating FABP4 in all patients one week post-surgery; thus it is possible that acute changes in intracellular FABP4 may precede a decrease in circulating FABP4. However, we did observe a decrease in those with the highest levels of FABP4 (>100 ng/mL) preoperatively. This subset of patients may be a cohort that significantly benefits from undergoing bariatric surgery. While longitudinal studies are required to assess this possibility, circulating FABP4 has been observed to decrease one year following RYGB (38). Nevertheless, the impact of bariatric surgery in ameliorating insulin resistance is also evident in patients in which serum FABP4 levels do not change acutely. Therefore, the acute decrease in intracellular levels of FABP4 in adipose tissue may still have a significant role underpinning the improvement following bariatric surgery.

Increased levels of FABP4 have been associated with increased risk of cardiovascular disease and T2DM (39, 40). Mechanistically, our group has identified the loss of FABP4 leads to increased expression of UCP2 (15). Increased UCP2 was proven critical for ameliorating oxidative stress, which favors chronic inflammation and endoplasmic reticulum (ER) stress. Given the importance of chronic inflammation and ER stress in the development of insulin resistance, we propose that decreased FABP4 and increased UCP2 expression in SAT may be responsible for the acute improvement in insulin sensitivity

following bariatric surgery. Indeed, a previous report from Ueda *et al.* has shown that one week following RYGB, 8-iso-prostaglandin F2 alpha, a serum marker of oxidative stress, is dramatically decreased (41). Consistent with this observation, we assessed cysteine oxidation in adipose tissue and also observed a consistent reduction.

It is worth noting that one limitation of this study is that we are unable to rule out the effects of changes in FABP4 expression of visceral adipose tissue, which is considered highly metabolically active. It has previously been shown that *FABP4* mRNA levels in SAT of obese patients are significantly higher than that of a lean cohort (42). Furthermore, in the obese population, *FABP4* mRNA levels were demonstrated to be greater in SAT than VAT. Protein levels were not measured. Thus it may be argued that because SAT is much more abundant than visceral adipose tissue, it could have a significant role in mediating part of the metabolic improvement observed following bariatric surgery.

Further work is required to understand the therapeutic potential of these changes along the continuum of weight gain and insulin resistance to weight loss and insulin sensitivity. As such, we are currently working to identify the responsible pathway(s) linking surgery to adipose tissue, specifically focusing on how PPARγ and FABP4 decrease and contribute to improved outcomes in metabolic disease. It is also imperative for the prescribing clinician to be cognizant that continued

use of TZDs after bariatric surgery may reduce the metabolic benefit afforded to post-surgical patients.

MATERIALS AND METHODS

Study Subjects. The University of Minnesota and St. Cloud Hospital Institutional Review Boards approved all investigations and informed consent was obtained from each participant. Twenty consecutive patients > 21 years of age and BMI ≥ 35.0 kg/m^2 undergoing VSG were recruited and informed consent obtained. Abdominal SAT biopsies from twelve of the VSG patients were processed for tissue analysis. The details of the study methods of the twenty-seven patients recruited and randomized to RYGB (*n*=13; 3 males and 10 females) or HC Diet (*n*=14; 4 males and 10 females) have been published (18). Briefly, criteria included men and women older than 21 years of age with a BMI ≥ 35.0 kg/m^2, and meeting criteria by ADA standards for pre-diabetes or T2DM.

Clinical data. Demographic data on sex, age, and T2DM were collected for obese patients at the time of surgery and seven days following bariatric surgery. Weight and height were measured immediately prior to surgery (preop) and during the postoperative visit. Body mass index (BMI) was calculated as weight (kg) divided by height (m^2).

Surgical Procedures. Details of the surgical procedures have been previously described(18, 21). With regards to the RYGB, a 20-30 mL vertically oriented gastric pouch was constructed using 3.5 mm staples. A 10-12 mm gastrojejunostomy was created in an antecolic, antegastric fashion, with the

Roux limb measuring approximately 150 cm from the gastrojejunostomy. For the laparoscopic VSG, a 4.8 mm stapler load was used to divide the greater curvature of the stomach 5 cm from the pylorus and remaining 3 cm from the *angularis incisura*. 3.5 mm stapler loads were fired thereafter progressing up to the angle of His to complete the VSG.

Tissue Sampling and blood collection. As detailed previously, subcutaneous white adipose tissue samples and blood specimens were obtained from patients on the day of intervention and seven days later. Approximately 2-3g of fat was obtained from each subject and immediately frozen with liquid nitrogen in 0.1-0.2 g aliquots, and stored at -80° C until further study. Fasting blood specimens were collected prior to intervention and one year post-intervention in patients in DSS.

Assessment of DM medication score and insulin resistance. The use of a diabetes medication score has previously been described (27). Fasting plasma glucose and plasma insulin levels were obtained at the time of initiation of intervention, and seven days later. In each subject, the degree of insulin resistance was estimated by Homeostasis Model of Assessment for Insulin Resistance (HOMA-IR) according to the method described by Matthews *et al.*(43) Glycated hemoglobin or Hemoglobin A1c (HbA1c) was determined preoperatively on all patients.

Collagenase Digestion. After fat was obtained, it was immediately minced and digested with type I collagenase in Krebs-Ringers-HEPES (KRH) buffer supplemented with 10 mg/ml bovine serum albumin (BSA). After incubation at 37°C for one hour, the mixture was filtered with a cell strainer (100-µm-pore-size nylon; Falcon) to remove undigested tissues. The SVF was collected by centrifugation at 500 x g for 10 minutes and both fractions, the floating primary adipocyte and the SVF were washed, and TRIzol © (Invitrogen, Carlsbad, CA) reagent was used for RNA isolation.

Real-time PCR. Total RNA was extracted from approximately 0.3 g of adipose tissue using Trizol © (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After DNase treatment, cDNA was synthesized using iScript cDNA synthesis kit (BioRad, Hercules, CA). Relative quantification of mRNA was performed by RT-PCR using iQ SYBR green Supermix and the MyiQ detection system (BioRad, Hercules, CA). Human primers for target genes are listed in Supplementary Table 2. Gene expression data are expressed as arbitrary units normalized to the reference gene hTBP.

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')
TBP	AGCGGTTTGCTGCGGTAATC	ACTGTTCTTCACTCTTGGCTCCTG
PPARγ	ACCAAAGTGCAATCAAAGTGGA	ATGAGGGAGTTGGAAGGCTCT
FABP4	CTGGTGGTGGAATGCGTCATGA	CTGGTGGTGGAATGCGTCATGA
ATGL	AGACGCCTCCATTACCACTG	AAGACCCCTCTTGGCAACTG
PDE3B	TCAACTCCATTTCCACCTCC	GACCGTCGTTGCCTTGTATT
Perilipin 1	CCATGTCCCTATCAGATGCCC	CTGGTGGGTTGTCGATGTC
Adiponectin	GGTCTCGAACTCCTGGCCTA	TGAGATATCGACTGGGCATGGT
CD36	CTTTGGCTTAATGAGACTGGGAC	GCAACAAACATCACCACACCA
CIDEC	GAGGTCCAACGCAGTCCAGCTG	GTACGCACTGACACATGCCTG
SCD1	TCTAGCTCCTATACCACCACCA	TCGTCTCCAACTTATCTCCTCC
LPL	TCATTCCCGGAGTAGCAGAGT	GGCCACAAGTTTTGGCACC
UCP2	CCTCATGACAGATGACCTCC	TGTATCTCGTCTTGACCACG

Supplementary table 2 - Real-time qPCR primer sequences.

Immunoblotting. Immunoblotting was performed as previously described (15). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. After a blocking step, membranes were incubated with primary antibody overnight at 4°C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 h and visualized using Odyssey infrared imaging (Li-Cor Biosciences, Lincoln, NE). The antibodies used were anti-PPARγ (Santa Cruz, Dallas, Texas), anti-FABP4, anti-CD36 (R&D Systems, Minneapolis, MN), anti-Perilipin 1 (American Research Products, Waltham, MA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO).

Mitochondrial isolation. Mitochondrial isolation was carried out as previously described(15). Adipose tissue was minced in ice-cold mitochondrial isolation buffer (20 mM Tris pH 7.4, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) and supplemented with protease inhibitors. The tissue was then further homogenized with 15 strokes of a Dounce homogenizer and centrifuged at 700 x g for 10 minutes at 4° C. Avoiding the lipid layer, the supernatant was transferred to a fresh tube and centrifuged at 10,000 x g for 15 min at 4° C.

Dimedone labeling of sulfenic acids. Mitochondria pellets were solubilized using sonication in radioimmunoprecipitation assay (RIPA) buffer containing

protease inhibitors, 1 mM dimedone, 10 mM iodoacetamide, 10mM N-ethylmaleimide, and 200 units/mL catalase to metabolize intracellular hydrogen peroxide. The lysates were incubated for 1 hr on ice followed by the addition of Laemmli protein sample buffer and the proteins were separated on an SDS-PAGE gel for cysteine oxidation (sulfenic acid) detection (44).

ELISA measurement of serum FABP4. Serum FABP4 was measured with human FABP4 Quantikine ELISA kit from R&D systems (#DFBP40) according to the manufacturer's instructions.

Statistical analysis. The data are expressed as means +/- standard error of the mean. Statistical analysis was performed using unpaired Student's t-test when comparing between groups and paired Student's t-test to compare pre- and post-surgical measures within groups. Unadjusted Pearson correlations were calculated to evaluate relations between HbA1c and target of interest, and changes in FABP4 and hormone of interest. Data with p-values less than 0.05 were considered statistically significant, unless otherwise stated.

Acknowledgement

This work was supported by the American Diabetes Association (ADA 7-11-ST-01), NIH DK053189 to DAB and the Minnesota Obesity Center (NIH P30DK050456). We thank the members of the Bernlohr laboratory, the University Minnesota Department of Surgery Division of Gastrointestinal and Bariatric Surgery, and the CentraCare Bariatric Center (St. Cloud, MN) for their assistance during the preparation of this manuscript. We would also like to thank the Minnesota Supercomputing Institute.

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CHAPTER FIVE

Conclusions and Perspectives

Hongliang Xu wrote this chapter in the entirety

Obesity has become a worldwide health concern especially in the western world. Currently almost one third of the American population are obese and are at higher risk of developing metabolic syndromes such as insulin resistance, fatty liver disease, diabetes, atherosclerosis, cardiovascular disease, and even some types of cancers (1, 2). While these diseases cost the health care system billions of dollars every year, a large percentage of the obese population is still not getting proper medical treatment, leading to further economic burdens.

One obvious outcome of obesity is adipose tissue expansion, which is accompanied with increases in both adipocyte size and immune cell recruitment (3). Indeed, one classical hallmark of obesity is the development of chronic tissue inflammation and insulin resistance (4, 5). Immune cell infiltration was thought to be initiated by increased secretion of inflammatory adipokines, such as MCP1, TNFα, and saturated fatty acids. In addition to the adipose tissue, these immune cells upon activation, contribute to the development of chronic inflammation and insulin resistance in other peripheral tissues, such as liver and skeletal muscle (6).

In addition to inflammation, increased oxidative stress, activation of ER stress pathways, and increased mitochondrial dysfunction have all been suggested to be involved in obesity and insulin resistance (7-10). Just to make this case more complicated, these factors contributing to insulin resistance are often mutually causal to each other, indicating synergistic effects of this phenomenon. As evidence to this synergy, rodents put on high fat diet to induce obesity related

syndromes exhibit increased oxidative stress as well as increased inflammation (11). Also, a number of studies have shown increased oxidative stress can trigger inflammatory pathway activation, which further induces more ROS production to form a feed forward loop (12). For instance, inflammatory lipid stimulation or increased energy loading in mitochondria lead to the production of more pathological levels of ROS, which exceeds the reducing power of the cell. This often leads to large-scale cysteine or methionine residue oxidation, altering the activity of the cellular signaling network. Pathways involved in this network include, but not limited to, glucose metabolism, TCA cycle, NF-κB pathway, HIF pathway, MAPK pathway, and mTOR pathway. The collective effect of these pathway alternations will eventually lead to mitochondrial dysfunction, ER stress, and inflammation.

Indeed, many drugs developed to treat diabetes converge on the impact of the whole network. For instance, AMPK activator, metformin, has been shown to protect cells from damage caused by oxidative stress by increasing autophagy (13). An example of this is aging, which is accompanied by potentiated oxidative stress and decreased AMPK activity (13). Furthermore, the role of AMPK in suppression of inflammation and mitochondrial dysfunction, as it relates to disease, is observed in a number of different cell types (14, 15). TZDs, which were identified as PPARγ ligands, have very potent effects on suppressing oxidative stress and reducing inflammation in macrophages (16, 17). Another important drug target for treating diabetes are sirtuins - class three-histone

deacetylase (18). Resveratrol, as well as its derivatives, has been identified as potent activator of both SIRT1 and SIRT3 (19, 20). Treatment of cells with resveratrol reduces both oxidative stress and inflammation, and may simultaneously improve mitochondrial function (21). The miraculous converging of these drugs for treating diabetes all indicates the importance of the redox-mitochondria-ER-nucleus network. Understanding of this network, instead of focusing on individual pathways or protein targets, has the potential to be instrumental in developing new drugs and therapies for treating diabetes. One such example is the combined use of metformin and salicylate, which has shown greater efficacy in treating diabetes (22). Also, the interconnected relationship of the pathways linked to obesity can provide inspirations to treat other relevant syndromes. One such example is PPARγ agonists that can also be used to treat inflammatory bowel disease, due to the immune-suppressive effect of PPARγ activation (23). Future work and understanding of pathways already in the network, or going to be connected to the network, can be helpful guidelines for developing more specific targets to individual patients, as well as providing new recipes for adjuvant treatments of the obesity related syndromes.

In my thesis research, we identified a FABP4/aP2-PPARγ-UCP2 (SIRT3)-redox axis, which is a good example of lipid metabolism by regulating mitochondrial ROS production affecting ER stress and inflammation. Loss of FABP4/aP2 in macrophages leads to an increase of intracellular monounsaturated fatty acids (MUFAs). In independent experiments we have shown that treatment with

MUFAs, which is a major component of Mediterranean diet, leads to increase of both UCP2 and SIRT3. Interestingly, dietary supplement of MUFAs has been shown to induce the expression of PPARγ in adipose tissue, with concomitant reducing adiposity and inflammation (24). In FABP4/aP2 deficient macrophages, increased MUFAs are accompanied with increased expression of PPARγ as well as PPARγ targets. By stimulating PPARγ activity with its agonist in macrophages, UCP2 expression was also increased, indicating PPARγ connects MUFAs to UCP2 expression in macrophages. By putting this story together, we propose loss of FABP4 leads to a specific increase of MUFAs pool, which may directly or indirectly serve as natural ligands of PPARγ to induce UCP2 expression. In doing so, we have made a novel connection of lipid metabolism and signaling in regulating mitochondrial function.

UCP2 is a well-defined ROS suppressor (25). Increased UCP2 activity decreases mitochondrial-generated hydrogen peroxide, which is an important signaling molecular to trigger downstream effectors in both cytosol and ER that affects the cellular redox balance. From this perspective, mitochondrial serve as a signaling hub that sense intracellular or extracellular signals, process the information, and adjust its own metabolic processes to send further signals to other organelles.

Interestingly, mitochondrion is also an important regulator of calcium homeostasis and metabolite availability. For instance, TCA intermediates such as succinate, fumarate, α-ketoglutarate (α-KG), and acetyl-CoA are important regulators of both cellular signaling and chromatin modifications (26). Succinate

and α-KG are important regulators of HIF activity, which can affect glucose metabolism (27). Furthermore, succinate and α-KG can regulate a number of demethylase activity and affect both histone and DNA methylation status(26). It will be interesting to test if UCP2, by changing the redox status of mitochondrial can affect the metabolites processing both in mitochondria and cytosol. Indeed, some groups have suggested UCP2 can uptake calcium from ER, while other's shown data support UCP2 as a C4 transporter (28, 29). While, more *in vivo* data is needed to verify these statements, it does provide hints that suggest UCP2 is an important regulator of energy source utilization and metabolite accumulation.

As an important mitochondrial redox regulator, UCP2 knock out macrophages are more proinflammatory and produce more inflammatory cytokines (30). With these current pieces of data, UCP2 should be accepted as an orchestrator of cellular metabolism network in order to adjust the cells inflammatory status.

In my thesis, we have also shown convincing data that the FABP4-UCP2 axis holds true in the human bariatric surgery model. Although bariatric surgery is to-date the most potent treatment in almost any diabetic related complication, the mechanism has been elusive with most researchers focuing on gut or gut related hormonal signaling. Our work offers a new perspective to understand the mechanistic reason for early-on benefits of the surgery, which may be pertinent in long-term metabolic improvements. In our model, the upregulation of UCP2, as a result of declined FABP4 expression in adipose tissue, will shift the redox and metabolic status in adipose tissue, reduce lipolysis and increase fatty acid

consumption. However, more work is still required to further characterize and delineate pathways involved and affected by changing UCP2 expression in adipose tissue. Nevertheless, the current work sheds lights into areas of great clinical relevance that were previously under appreciated. Given the significant percentage of patients not suitable to undergo surgery, and the surgical complications that often arise, defining the mechanism of improved metabolic health from bariatric surgery will have a tremendous impact on diabetes care.

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BIOGRAPHICAL SKETCH

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NAME **Xu, Hongliang**	POSITION TITLE **Research Assistant**
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Minnesota		2011-present	Chronic metabolic disease
Capital Normal University	Master in Genetics	2008-2011	Molecular Genetics
Xi'an Polytechnic University	Bachelor in Bioengineering	2004-2008	Bio-engineering

Personal Statement

Obesity is associated with increased endoplasmic reticulum stress and systemic insulin resistance. Loss of adipocyte fatty acid binding protein (AFABP) uncouples obesity from ER stress and inflammation. My work shows that deletion of AFABP in macrophages results in elevated uncoupling protein 2 (UCP2) expression. UCP2 silencing in AFABP deficient macrophages negated the protective effect of FABP loss and increased ER stress in response to palmitate or lipopolysaccharide. Interestingly, in human subcutaneous fat from patients going through bariatric surgery, decreased expression of AFABP is accompanied with a significant increase of UCP2, which could mediate the overall improvement of patients after surgery. My current work is trying to use macrophage UCP2 knock out mouse model to demonstrate UCP2 expression in macrophages is indicative of bariatric surgery efficiency.

Positions (ending with present position)

2008-2011 Research Assistant, Graduate Program in Genetics, Capital Normal University.
Advisor, Dr. Yingkao Hu

2011-present Research Assistant, Graduate Program in Biochemistry, Molecular Biology & Biophysics
Advisor, Dr. David Bernlohr

Honors and Awards

2009-2010	First Class Scholarship for Graduate Students, Capital Normal University
2010-2011	“Bai Chuan Cup” Award for Excellent Graduate Student
2010-2011	Excellent Graduate Student Award of 2011, Capital Normal University
2012-2013	Armstrong-Pothapragada Fellowship
2013-2014	Cargill fellowship
2014-2015	Charles W. Carr & William J. Peterson Award
	Maurice B. Visscher Symposium 2015 Yong Investigators’ Competition Award
	Cargill Fellowship
2015-2016	Bacaner Research Award
	Thomas Reid Award
	Cargill Fellowship

Peer Reviewed Publications

Hongliang Xu, Yaxuan Li, Yueming Yan, Ke Wang, Ya Gao, Yingkao Hu*: Genome-scale Identification of Soybean BURP domain-containing genes and their expression under stress treatments. *BMC Plant Biology* 2010, **10**:197

Xinglu Yang, **Hongliang Xu**, Wenhui Li, Le Li, Jinyue Sun, Yaxuan Li, Yueming Yan, Yingkao Hu*: Screening and identification of seed-specific genes using digital differential display tools combined with microarray data from common wheat. *BMC Genomics* 2011, **12**:513

Guangjun Yin, **Hongliang Xu**, Shuyang Xiao, Yajuan Qin, Yaxuan Li, Yueming Yan, Yingkao Hu*. The large soybean (Glycine max) WRKY TF family expanded by segmental duplication events and subsequent divergent selection among subgroups. *BMC Plant Biology* 2013, **13**:148

Guangjun Yin, **Hongliang Xu**, Jingyi Liu, Cong Gao, Jinyue Sun, Yueming Yan, Yingkao Hu*. Screening and identification of soybean seed-specific genes by using integrated bioinformatics of digital differential display, microarray, and RNA-seq data. *Gene* 2014, **2**: 177-186

Hongliang Xu, Ann V. Hertzel, Kaylee A. Steen, Qigui Wang, Jill Suttles, David A. Bernlohr*. Uncoupling lipid metabolism from inflammation through FABP-dependent expression of UCP2. *Molecular Cellular Biology* 2015, **35**(6): 1055-1065

Cyrus Jahansouz, **Hongliang Xu**, Ann Hertzel, Federico Serrot, Nicholas Kvalheim, Abigail Cole et al. Bile acids increase independently from Hypocaloric restriction after bariatric surgery. *Annals of Surgery* 2015, ahead of print

Hongliang Xu, Ann V. Hertzel, Kaylee A. Steen, David A. Bernlohr. Loss of fatty acid binding protein 4/aP2 reduces macrophage inflammation through activation of SIRT3. *Molecular Endocrinology* 2016, 30(3):325-334